REGULATION OF ANTIGEN PRESENTATION

IN DENDRITIC CELLS

HUANG DACHUAN

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

NATIONAL UNIVERSITY OF SINGAPORE

2008

ACKNOWLEDGEMENTS

Deepest appreciation to the following:

My supervisor, Dr Wong Siew Heng for this opportunity to pursue research and for his patient guidance during my study;

A/Prof Sim Tiow-Suan and A/Prof Vincent Chow for their concern and guidance;

Members of flowcytometry team, Mr Chan Yue Ng, Ms Nalini Srinivasan and Ms Thong Khar Tiang for their help in setting up the FASCAN protocol;

Ms Tan Suat Hoon in EM unit for her help with electron microscopy;

All the staff of the department especially Ms Josephine Howe LC, Mr Ng Han Chong, Mr Ramachandran NP, Ms Siti Masnor and Ms Geetha Baskaran;

Ms Tan Yinrou and Dr Low Choon Pei for their time spent in helping to proofread;

My lab mates, Dr Ho Lip Chuen, Mr Sunny, Mr Ray, Ms Tarika and Ms Kershin and Ms May Ling for their encouragement, friendship and help;

My dearest friends Dr Teleguakula Narasaraju, Dr Ye Enyi, Dr Kiew Shih Tak, Dr Susan Amy and Dr Peter Zhu for being there always through the ups and downs;

My family for being there for me always and supporting me through these years.

TABLE OF CONTENTS

SUMMARY

Dendritic cells (DCs) are able to stimulate T cell and initiate immune responses via the antigen presentation pathway which is regulated by major histocompatibility complex class II (MHC class II) and its accessory molecules such as invariant chain CD74 and MHC class II like molecule H2-M. The expression of these molecules is mainly controlled by class II transactivator (CIITA). CIITA is a non-DNA binding co-activator, and serves as a platform for the recruitment of various *trans*-factors which are require for successful transcription of MHC class II and its accessory molecules. Here, we identified and described the function of a novel isoform of CIITA, DC-expressed caspase inhibitory isoform of CIITA (DC-CASPIC). DC-CASPIC is expressed in immature DCs and its protein expression is up-regulated upon DC maturation. In mature DCs, DC-CASPIC localises to mitochondria and interacts with caspases thereby inhibiting caspase activities. Since nitric oxide synthase-2 (NOS2) is a substrate for caspases, DC-CASPIC thus inhibits the caspase-dependent degradation of NOS2 and induces nitric oxide (NO) synthesis in maturing DCs. Furthermore, similar to lipolysaccharide-treated DCs, DCs over-expressing DC-CASPIC enhance MHC class II, CD80 and CD86 cell surface expression and stimulate T cell proliferation. Taken together, our results strongly suggest that DC-CASPIC is one of the key molecules that regulate NO synthesis and antigen presentation during maturation of DCs.

Next, we dissected the detailed mechanism of NOS/NO-enhanced antigen presentation during maturation of DCs. We reported that in immature DCs, the NO donor and the over-expression of either NOS2 or NOS3 alone could induce the cell surface expression of MHC class II, CD80 and CD86. Consistently, NO

donor-treated immature DCs were capable of enhancing T cell proliferation *in vitro* in the absence of lipolysaccharide. Interestingly, NOS2 interacted with CD74, and the degradation of CD74 by caspases in immature DCs was inhibited upon treatment with the NO donor. Since the trafficking of MHC class II is CD74-dependent, the increase in cell surface localisation of MHC class II in maturing DCs could be partly due to the increase in CD74 protein expression in the presence of NOS2 and NO. These studies may provide a novel platform to enhance the antigen presentation ability of DCs and to develop or design potent vaccines against infectious diseases and cancers.

FIGURES AND TABLES

List of figures

List of tables

LIST OF ABBREVIATIONS

AEP	Asparagine endopeptidase
AP1	Activator protein 1
AP-1	Adaptor protein-1
APCs	Antigen presentation cells
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BLS	Bare lymphocyte syndrome
CARD	Caspase recruit domain
cDCs	Conventional dendritic cells
CFSE	Carboxy fluoroscein succinimidyl ester
CIITA	Class II transactivator
CLIP	Class II associated invariant chain peptide
CMK	Chloromethylketone
CREB	cAMP response element binding
Cy3	Cyanine dyes 3
DAPI	4', 6-diamidino-2-phenylindole
DC-CASPIC	DC-expressed caspase inhibitory isofrom of CIITA
DCs	Dendritic cells
DED	Death effector domain
DMEM	Dulbecco's minimal eagles medium
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting

FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FMK	Fluoromethyl-ketone
GM-CSF	Granulocyte monocyte colony stimulating factor
GST	Glutathione *S*-transferase
HRP	Horse radish perooxidase
IFN	Interferon
IPTG	Isopropyl-*β*-D-thiogalactopyranoside
$_{L}$-NMMA	N^{G}-monomethyl-$_{L}$-arginine
LPS	Lipolysaccharide
LRRs	Leucine-rich repeats
MAC	Myristoylated alkaline-rich protein kinase C substracte
MAVS	Mitochondrial antiviral signalling
MAPK	Mitogen-activated protein kinase
M-CSF	Monocyte colony stimulating factor
MIF	Migration inhibitory factor
MLR	Mixture lymphocyte reaction
MHC CLASS II	Major histocompatibility complex class II
LB	Lysogeny broth
NDB	Nucleotide binding domain
NFY	Nuclear factor Y
NLS	Nuclear-localisation signal
NO	Nitric oxide
NOD	Nucleotide-binding oligomerisation domain
NOS	Nitric oxide synthase

ORF	Open-reading-frame
RIG	Retinoic acid-inducible gene
RIP	Regulated intramembrane proteolysis
RFX	Regulatory factor X
RT-PCR	Reverse transcriptional polymerase chain reaction
PBS	Phosphate buffer saline
pDCs	plasmacytoid dendritic cells
PMSF	Phenylmethylsulfonyl fluoride
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNP	Sodium nitroprusside
TEC	Thymic epithelial cells
TGN	*trans*-Golgi network
Tip DCs	Inflammatory dendritic cells
TNF	Tumor necrosis factor
TLR	Toll like receptor
WASP	Wiskott-Aldrich syndrome protein

CHAPTER 1 OVERVIEW

Dendritic cells (DCs) consist of a heterogeneous population of cells that accomplish key functions in the immune system, including the establishment of a central tolerance in the thymus, the maintenance of self-tolerance in the periphery and the initiation and regulation of an adaptive immune response (Banchereau *et al.*, 2000). These functions of DCs are conferred by their ability to internalise, process and present antigens to T cells. The presentation of peptides to the antigen receptor on $CD4^+$ T helper lymphocytes is mediated by the major histocompatibility complex class II molecules (MHC class II), invariant chain CD74 and MHC class II like molecule H2-M as well as other regulatory molecules. In this pathway, MHC class II and CD74 are assembled in the endoplasmic reticulum (ER) and transported to the endosomal membrane system. In the endosomal membrane system, CD74 (bound to the peptide-binding groove of the MHC II molecule) is degraded into a short peptide called class II associated invariant chain peptide (CLIP). Under the influence of H2-M function, CLIP is released and replaced by the antigenic peptides in the endosomes. The MHC class II and antigenic peptides form complexes, and then are transported to the cell surface and presented to T cells.

Because $CD4^+$ T cells only recognise antigen-derived peptides bound to MHC class II molecules but not free antigenic peptides, MHC class II molecules play a crucial role in the antigen presentation pathway. Accordingly, the lack of MHC class II at the cell surface results in an autosomal and recessive severe combined immunodeficiency called the bare lymphocyte syndrome. Moreover, their inappropriate expression ~~on~~ in target tissues results in organ-specific

autoimmunity (Reith and Mach, 2001). Hence, the expression of MHC class II in all antigen-presenting cells must be tightly controlled. In DCs, MHC class II is regulated (1) at the transcriptional level by the MHC class II transcription activator (CIITA) (Ting and Trowsdale, 2002), and (2) at the translational/post-translational level by its chaperone, CD74 (Cresswell, 1994).

The transcriptional regulation of MHC class II mainly occurs at its conserved up-stream sequence that stretches through 75 nucleotides containing and contains several cis-elements such as X box, Y box and S box. In this region, regulatory factor X (RFX) binds to the X and S boxes, whereas nuclear factor Y (NFY) binds to the Y box which is composed of the CCAAT sequence. Activator protein 1 (AP1), X2 binding domain (X2BP) and cAMP response element binding protein (CREB) interact with the X2 box. However, the presence of these transcription factors alone is not sufficient to initiate the transcription of MHC class II. The transcription of MHC class II takes place only after CIITA is recruited to provide a platform for the binding of these factors. This CIITA-containing complex, which is called MHC class II enhanceosome, is thus essential for the expression of MHC class II. It also takes part in the regulation of CD74 and H2-M transcription (Reith and Mach, 2001) (Figure 1).

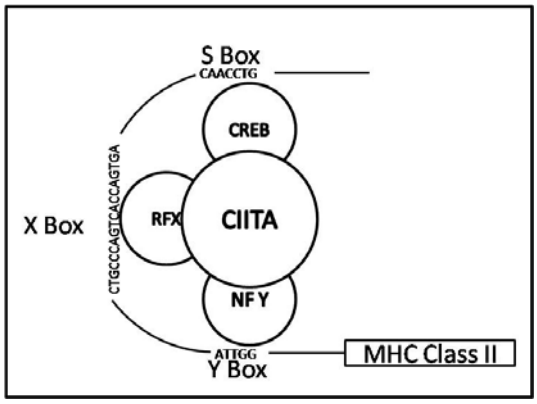


Figure 1: Schematic of MHC class II enhanceosome

Unlike RFX and NFY, the expression of CIITA is highly regulated by four different promoters in humans (pI, pII, pIII, pIV) depending on the cell types (Figure 2). The product of pII is absent in mice; pIV is mainly expressed in thymic epithelial cells (TEC) and non-hematopietic origin cells; pIII is a lymphoid promoter that is active in B cells and plasmacytoid DCs (pDCs); pI is specifically activated in DCs and its translation product is called CIITA type I, or DC-CIITA (Muhlethaler-Mottet *et al.*, 1997). Nickerson *et al* (2001) reported that in contrast to the translated products from pIII and pIV, the CIITA protein synthesised from pI contained an extra caspase recruitment domain, namely the CARD domain (Figure 2). Interestingly, unlike other CARD-containing proteins, DC-CIITA does not interact with caspases. However, the CARD-like domain specific to DC-CIITA has a higher transactivation activity when compared to other forms of CIITA, which do not contain CARD-like domain. The molecular mechanisms that underlie the function of the CARD-like domain remain largely unknown. It was hypothesised that DC-CIITA's CARD-like domain might recognise the CARD on unidentified proteins, possibly transcription factors, or proteins that coordinate with the CIITA complex to enhance the transactivation of the MHC class II gene (Nickerson *et al.*, 2001). The elucidation of the function of the CARD-like domain will be particularly important in the understanding of the function of DCs because it has been shown that DC-CIITA expression in DCs is driven mainly by pI, and that DC-CIITA transcripts are by far the most abundant compared with pIII and pIV products. mRNA transcripts in immature DCs (Leibundgut-Landmann *et al.*, 2004).

As an initial step for this study, the CARD domain of DC-CIITA was cloned by RT-PCR with specific primers. Interestingly, in addition to the expected

DC-CIITA PCR product, an extra and larger PCR product was generated. Its sequence revealed a new open reading frame (ORF) that can be translated into a novel protein composed of a CARD domain of DC-CIITA and a 28-amino acid tail that has no significant homology to any known proteins/peptides in the Entrez database. This novel protein was named DC-CASPIC.

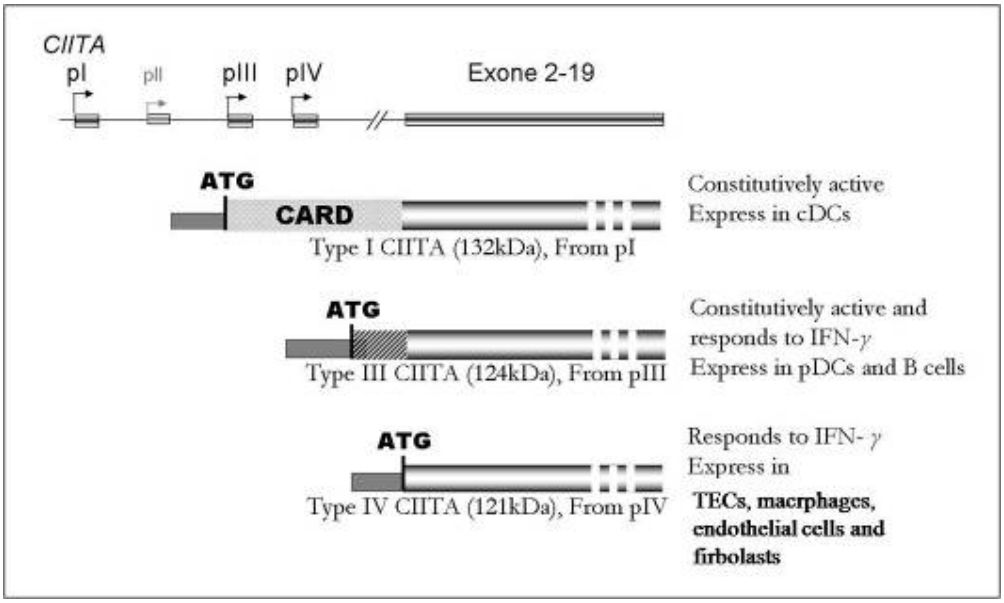


Figure 2: Modular structure of regulatory region of gene encoding CIITA

The subsequent functional study on DC-CASPIC revealed that increase of NO concentration resulted in DC-CASPIC over-expression in DCs-inhibited caspase activity, and elevated cell surface expression of MHC class II in DCs. In order to successfully transport to cell surface and present the antigenic peptide to response T cells, MHC class II needs the help from its chaperone, CD74. CD74 stabilises the MHC class II molecules, prevents premature peptide loading and target MHC class II molecules to late endosomal compartments. In addition, CD74 also controls the proteolysis of H2-M protein which is required for efficient MHC class II antigenic peptide loading (Pierre and Mellman, 1998; Pierre *et al.*, 2000).

Unlike CIITA, which is controlled by different promoters at transcriptional level, CD74 expression is highly regulated via stepwise proteolysis events. Full-length CD74 (p31) bound to the MHC class II molecule is cleaved by proteases to give rise to p22, and then p10. P10 is further digested into the MHC class II-associated invariant chain peptide CLIP. CLIP then dissociates from MHC class II with the help of H2-M, which facilitates the exchange of CLIP for the antigenic peptides (Figure 3) (Denzin and Cresswell, 1995; Hsing and Rudensky, 2005). Despite the identification of the various degradation products of CD74, the exact pathway of CD74 degradation remains unclear. Although the endoproteases responsible for the initiation of CD74 proteolysis have been reported to be a leupeptin-insensitive protease, its identity is still unclear. For example, asparagine endopeptidase (AEP) has been earlier reported to cleave CD74 in B cells (Manoury *et al.*, 2003). However, it was later found to be dispensable in a study using AEP-knockout mice (Matza *et al.*, 2003; Maehr *et al.*, 2005). Pierre and Mellman proposed that cystatin C might predominantly control the regulation of CD74 protein degradation via the inhibition of activity of cathepsin S which was essentially involved in generating CLIP (Pierre and Mellman, 1998). Nevertheless, no co-localisation of cystatin C with the MHC class II compartments has been detected in either immature or mature DCs (Villadangos *et al.*, 2001). Moreover, knockout of the cystatin C gene has no effect on antigen presentation (El-Sukkari *et al.*, 2003). These discrepancies suggest that other proteases might be involved in CD74 degradation.

Previous work has shown that in immature DCs, a number of membrane trafficking-related proteins are degraded by caspases. In mature DCs, this caspase-mediated degradation is inhibited presumably by a mechanism closely linked to

the activity of nitric oxide synthase 2 (NOS2), that catalyses the production of nitric oxide (NO) in large amounts during maturation of DCs (Wong *et al.*, 2004; Santambrogio *et al.*, 2005). Our results also indicate that NOS2 and caspase may be involved in the regulation of CD74 proteolysis in DCs.

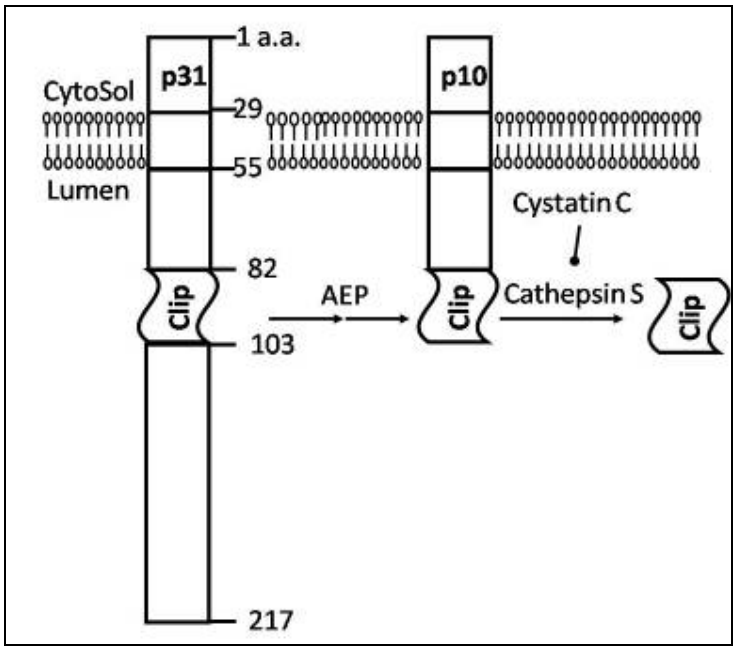


Figure 3: Schematic of CD74 degradation pathway

Therefore, the scope of this study includes: (1) Isolation of full-length DC-CASPIC cDNA; (2). Characterisation of DC-CASPIC expression profiles in mouse cell lines and tissues; (3) Analysis of DC-CASPIC function in DCs; (4) Identification of potential interaction partners of DC-CASPIC; (5) Identification of specific caspases that cleave CD74 in DCs; (6) Elucidation of NO-dependent mechanisms that regulate CD74 in DCs and (7) Analysis of the effects of CD74 degradation on the function of DCs.

CHAPTER 2 LITERATURE REVIEW

The mounting and regulation of effective adaptive immune responses heavily rely on antigen presentation. T cells are only able to recognise the pathogens/antigens that have been internalised by antigen-presenting cells (APCs) and processed into smaller fragments or peptides, which are then presented on the cell surface by MHC class I, II. MHC class I molecules are primarily essential for the presentation of cytosolic or viral antigens (Brigl and Brenner, 2004). MHC class I is also able to present exogenous peptides to T cells via the cross presentation pathway (Heath and Carbone, 2001). MHC class II molecules are predominantly expressed by antigen presenting cells (APCs) and they mostly present peptides derived from extracellular proteins to $CD4^+$ T cells. MHC class II molecules present antigens to $CD4^+$ T helper cells and thereby control the differentiation of B cells into antibody-producing B-cell blasts. Patients or mice failing to produce proper MHC class II–peptide complexes will not be able to raise sufficient antibody responses to infections. MHC class II is also important to modulate cytotoxic T-cell activation, autoimmune responses and other responses to pathogens or environmental antigens (Nuno Rocha *et al*, 2008). In the following literature review and study, the main focus will be on MHC class II mediated-antigen presentation pathway.

In principle, any type of cells that express surface MHC are able to present antigens to T cells (Malissen *et al.*, 1984). However, the efficiencies of antigen processing and presentation are vastly different (Mellman *et al.*, 1998). Therefore, those types of cells displaying high capability of antigen processing and

presenting are dubbed as professional APCs. Typically, professional APCs include B cells, macrophages and DCs. B cells mainly present the antigen to $CD4^+$ T cells. In turn, the $CD4^+$ T cells stimulate the B cells to grow into plasma B cells that produce antibodies. In fact, the antigen presentation by B cells is intimately linked to their primary function in antibody secretion rather to T cell response. Unlike B cells, macrophages are able to stimulate T cells of various specificities. However, macrophages are not very efficient in antigen presentation because the internalised materials are easily digested instead of being processed into antigenic peptides in its lysosomal compartments (Mellman *et al.*, 1998). In addition, compared with B cells and DCs, the amount of cell surface MHC class II is substantially lower on macrophage. This low level of MHC class II limits the macrophages' efficiency in antigen presentation and T cell stimulation *ex vivo* and *in vivo*. The major function of macrophages may not be in the adaptive immune system but in the innate immune system, by clearance of invading pathogens.

Unlike B cells and macrophages, antigen presentation appears to be the primary function of DCs. DCs are specialised for up taking, processing and presenting antigens to T cells via both MHC class I and class II pathway. And DCs are the only type of APCs which are capable of priming naïve T cells DCs (Randolph, 2001; Mempel *et al.*, 2004). The next section provides more details on the DCs (Table 1)

Table 1. Comparison of DCs, B cells and macrophages

	Dendritic cells	B cells	Macrophages
Antigen Internalization	+++	+	+++
Surface MHC class II	+++	++	+/-
Primary Function	Antigen presentation	Ab production	Pathogen clearance
Cross-presentation	+++	+/-	+/-
Naïve T cell priming	+++	+/-	-

2.1 Dendritic cells

2.1.1 Heterogeneity of DCs

The heterogeneity of DCs is indicated by their different precursors, life cycle, cell surface markers, localisation and different phenotypes (Villadangos *et al.*, 2007; Shortman and Naik, 2007). Based on their precursors, DCs in mice can be classified into three categories: (1) lymphoid-derived DCs (plasmacytoid DCs, pDCs), (2) myeloid-derived DCs (conventional DCs, cDCs) and (3) monocyte-derived DCs which are also called TNF-iNOS-producing DCs or inflammatory DCs (Tip DCs) (Ardavin, 2003).

pDCs are round, non-dendritic circulating DCs and exist in blood as well as in peripheral lymphoid organs. pDCs are distinguished from cDCs and TipDCs by expression of unique surface markers: CD123, CD303 and CD304, but not CD11c and CD14. In mouse, pDCs are also expressed B220 which is one of CD45 isofrom (Hideki Nakano, 2001). Upon stimulation of double strand RNA (dsRNA) or CpG DNA, pDCs are able to produce type I interferon, for example IFN-α and IFN-β, which are critical pleiotropic anti-viral compounds (Asselin-Paturel, 2001). The second category of DCs, lymphoid-tissue-resident cDCs can be divided into $CD8^-$ and $CD8^+$ subpopulations. Both subsets of cDCs can be induced undergo maturation. $CD8^-$ and $CD8^+$ cDCs have different immune functions. $CD8^-$ cDCs mainly elicit T helper 2 (Th2)-cell response, whereas $CD8^+$ induce strong Th1-cell response because of its high level of interlukin-12 (IL-12) production. Moreover, only $CD8^+$ cDCs are able to internalise apoptotic cells (Shortman and Naik, 2007).

The third category of DCs is TipDCs. TipDCs are characterised by the production of tumor necrosis factor (TNF) and NOS2. In addition, TipDCs can be distinguished from cDCs by their intermediate rather than high expression level of CD11c and CD11b, and by the absence of CD4 or CD8 expression (Shortman and Naik, 2007). TipDCs are circulating DCs but they can be recruited to inflamed tissue in a chemokine (C-C motif) receptor 2 (CCR2)-dependent manner (Miguel, *et al.* 2004). TipDCs are important during both bacteria and virus infection. In *L. monocytogenes* infection, TipDCs mediate an effective immune response at the time when other subsets of DCs are not competent to mount responses against infection. They are also predominant source of TNF and NOS2 and mediate innate immune response (Leon, *et al.*, 2007). In influenza infection, it is reported that TipDCs are required for the further proliferation of influenza-specific CD8+ T cells in the infected lung (Natalya, 2003).

In vitro, Tip DCs can be derived from bone marrow cultures in the present of granulocyte monocyte colony stimulating factor (GM-CSF). These bone marrow derived DCs (BMDCs) display phenotypic and functional characteristics that are similar to TipDCs *in vivo*. The BMDCs are positive for CD11c, MHC class II and negative for T and B cell markers. And they also have a high T-cell stimulation capacity (Ardavin, 2003). More importantly, they express high level of NOS2 and TNF during maturation (Berthier, 2000). Shen *et al*. (1997) also developed a TipDC clones (DC2.4) by introducing the GM-CSF gene into the C57BL/6 bone marrow cells, followed by infection with a retrovirus encoding *myc* and *raf* oncogenes. Similar to Tip DCs *in vivo* and BMDCs *in vitro*, they express DCs specific markers including MHC class I, MHC class II, CD80, CD86 and CD205, and express NOS2 and TNF production DCs upon stimulation. Both of

BMDCs and DC2.4 cell are able to internalise, process and present antigens to T cells as peptides in the context of MHC class I and MHC class II molecules (Akira Takashima, 2001 and Suzanne, 2008).

2.1.2 Maturation of DCs

There are three developmental stages in DCs: dendritic cell precursors, immature DCs and mature DCs. The two latter stages are closely related to the different immune functions of DCs. The immature DCs exhibit a high capability in antigen capturing and processing but low efficiency in T cell stimulation capability. During maturation, DCs undergo a series of changes: rearrangement of the cytoskeleton, reduction in phagocytic activity, acquisition of cellular motility, and increase in cell surface MHC class II, up-regulation of costimulatory molecules and secretion of cytokines. These changes work together to help DCs transit from immature antigen-capturing cells to mature antigen-presenting DCs (Banchereau *et al.*, 2000).

The maturation of DCs is initiated either by innate immunity maturation signals or by adaptive immunity maturation signals. The innate immunity maturation signals are included various pathogen-related molecules such as LPS, CpG RNA and double stranded RNA (dsRNA). The recognitions of these pathogen-related molecules are mainly attributed to Toll like receptors (TLR). In mouse, DCs express nine kinds of TLRs (from TLR1 to TLR 9) and each TLR has its own specific ligand. For example, TLR4 specifically interacts LPS and activates the NF-κB signalling pathway via the MyD88 dependent pathway (Banchereau *et al.*, 2000; da Silva Correia *et al.*, 2001). Besides, the maturation of DCs is also induced by immunity maturation signals including cytokines (CD40,

TNF-α, IL-6, IFN-γ, for example) and antigen-antibody and Fc Receptors (Ag-Ab IC) (Banchereau *et al.*, 2000).

Among all of these signals, LPS is one of most superior stimuli to induce DC maturation in regard to the concentration of co-stimulatory molecules and in regard to the kinetics of maturation. The percentage of mature DCs is quickly increasing and exceeded 95% only after 48 hours of stimulation. And the matured DCs exhibit a high concentration of cell surface markers especially co-stimulatory and MHC molecules (Matjaž Jeras, 2005). In this current study, the maturation of DCs was induced by LPS.

After the activation of transcriptional factors, the expression of many genes are up-regulated, including co-stimulatory molecules CD80 and CD86, as well as cytokines IL-12 and IFN-γ (Harris *et al.*, 2008). These molecules then enhance DCs so as to stimulate the activation of T cells. Besides the up-regulation of co-stimulatory molecules and the secretion of cytokines, the most fundamental change during the maturation of DCs is the redistribution of MHC class II that is, MHC class II moves from the intracellular membrane system where it is loaded with antigenic peptide, to the cell surface, whereby it presents the antigenic peptide to the T cells (Figure 4).

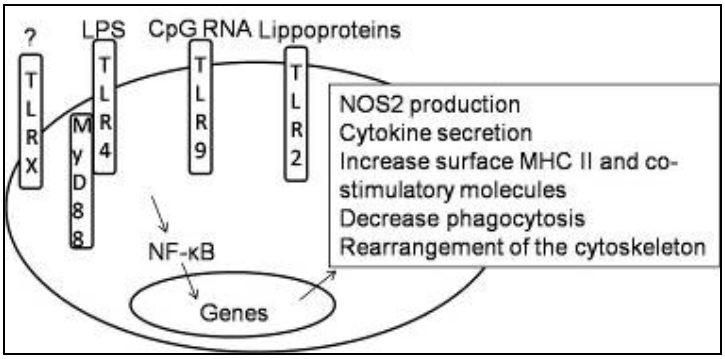


Figure 4: Maturation of DCs

2.2 Antigen presentation in DCs

DCs are able to induce either tolerance or immunity, depending on the subtype, location and developmental stage. Nevertheless, regardless of these factors, the immune activities of DCs hinge on antigen presentation and recognition by T cells in order to fulfil their immune activities. In other words, the functions of DCs are dependent on the antigen presentation pathway which is composed of three steps: antigen capture, antigen processing and antigen presenting.

Immature DCs have a high capability to capture antigens through three different approaches: macropinocytosis, receptor-mediated endocytosis and phagocytosis (Wilson and Villadangos, 2005). DCs can also internalise peptide-loaded heat shock proteins gp96 and Hsp70, which are ligands of TLR (Tsan and Gao, 2004). Differences in the mechanisms for capturing exogenous antigens offer opportunities for functional specialisations among different DCs subsets. For example, among cDCs, the DCs resident in thymus and spleen are most efficient at phagocytosing dead cells. Antigen receptors are also differentially expressed among DC subtypes. The differential expression of these receptors can provide each DC subset with distinct capacities to capture and initiate responses against specific pathogens. Nevertheless, no significant differences have been described in pinocytic activity among DC subsets (Villadangos and Schnorrer, 2007).

After the antigens are engulfed into DCs, they are transported into the endo-membrane trafficking system (such as the phagosome, endosome and lysosome), and are “chopped” into antigenic peptides with the help of various proteases. The antigenic peptides are then loaded into MHC class II molecules in

lysosomes or in MHC class II compartments (MIIC). The classic pathway of antigen presentation can be briefly outlined as follows: The MHC class II *αβ* heterodimers initially assemble in the endoplasmic reticulum with CD74 which acts as a chaperone to stabilise the heterodimers, prevent premature peptide loading and target MHC class II molecules to late endosomal compartments. CD74 is then partially dissociated through a series of proteolytic cleavage events, leaving a residual peptide (class II-associated invariant chain peptide, CLIP) occupying the peptide-binding groove of the MHC class II molecule. The final release of CLIP and its replacement with antigenic peptides is catalysed by H2-M (HLA-DM in humans), which is independently targeted to late endosomal compartments. The resulting MHC class II-peptide complex is then transported to the cell surface where it awaits interactions with antigen-specific T cells.

2.3 Regulation of antigen presentation in DCs

Antigen presentation is up-regulated during the maturation of DCs since immature DCs display at the surface an empty, peptide-receptive form of MHC class II, but only a few MHCII-peptide complexes, whereas mature immunogenic DCs express only high levels of long-lived MHC class II-peptide complexes (Santambrogio *et al.*, 1999;Villadangos *et al.*, 2001). This change is partially due to the deactivation of Cdc42, which belongs to the family of small Rho GTPases in mature DCs (Jaksits *et al.*, 2004). Cdc42 is hypothesised to transport MHC class II complexes from the cell membrane to lysosome-related MHCII compartments via the linker protein Wiskott-Aldrich syndrome protein (WASP) (Shurin *et al.*, 2005). Although immature and mature DCs express similar amounts of Cdc42 protein, only immature DCs contain detectable active form of Cdc42 that binds to GTP.

The loss of Cdc42 activity inhibits actin polymerisation in mature DCs, and leads to an accumulation of MHC class II on the cell surface (Wendy S. Garrett, *et al*, 2004). Antigen presentation by DCs is also efficiently regulated through the ubiquitination of MHC class II-β. In immature DCs, the MHC class II β chain is oligoubiquitinated after the degradation of associated CD74 in endosomes. The ubiquitination of the MHC class II β is inhibited in the LPS-induced mature DCs, resulting in the accumulation of MHC class II on the cell surface (van Niel *et al.*, 2006; Shin *et al.*, 2006). Antigen presentation is controlled not only by the intracellular distribution of MHC class II, but also by the surface expression of MHC class II.

2.3.1 Regulation of MHC class II expression by CIITA at the transcriptional level

2.3.1.1 CIITA

Earlier studies have established the critical role of the class II transactivator (CIITA) as a positive regulator or 'master controller' of MHC class II transcription (Ting and Trowsdale, 2002). A deficiency of CIITA or other factors, such as the RFX subunits, that are necessary for MHC class II transcription, results in the Bare Lymphocyte Syndrome (BLS) (Reith *et al.*, 2001). Unlike classical transcription factors, CIITA does not directly bind DNA; however it interacts with RFX5, NFY and CREB, and it has been proposed to function as a scaffold to promote the assembly of these transcription factors (Zhu *et al.*, 2000). MHC class II expression in thymic epithelial cells (TECs) and APCs strictly depends on the activation of the *CIITA* gene (Chang *et al.*, 1996) and the silencing of the *CIITA* gene abrogates MHC class II expression (Silacci *et al.*, 1994; van

den Elsen *et al.*, 2000). In addition, the expression of antigen presentation-related genes such as CD74 and H2-M are also under the control of CIITA (Chin *et al.*, 1997; Masternak and Reith, 2002). In DCs, CIITA is involved in negative regulation of IL-10 expression as well (Yee *et al.*, 2005).

Both mouse and human *CIITA* genes are encoded on chromosome 16. An analysis of mouse genomic DNA reveals that CIITA is encoded by 19 exons (Accolla *et al.*, 1986; Steimle *et al.*, 1994). Four different isoforms of human CIITA transcripts have been identified (Muhlethaler-Mottet *et al*, 1997). These isoforms are expressed under the control of different promoters (Figure 1). Three of these promoters (pI, pIII and pIV) are strongly conserved in the mouse *C2ta* gene but a mouse equivalent of pII is not known and therefore is not discussed here. Through the differential usage of pI, pIII and pIV, the *CIITA* gene determines the cell-type-specific cytokine-induced, and developmentally modulated MHC class II expression. The specificity of these promoters has recently been defined *in vivo* by generating mice carrying targeted deletions of the regulatory region of the *CIIta* gene. These results show that pIV is essential for CIITA expression in TECs and cells of non-hematopoietic origin after IFN-γ stimulation and the lack of pIV results in the absence of MHC class II expression in TECs, which leads to the abrogation of positive $CD4^+$ T cell selection (Leibundgut-Landmann *et al.*, 2004). *CIIta* pIII is a lymphoid promoter that is essential for the direction of CIITA expression in B cells and plasmacytoid DCs (pDCs). *CIIta* pI is a myeloid promoter that drives the constitutive expression of CIITA in conventional DCs (Figure 2).

All isoforms of the CIITA proteins contain transcription activation domain on the N-terminal, a centrally located nucleotide binding domain (NBD), and a carboxyl C-terminal region that consists of leucine-rich repeats (LRRs) (Steimle *et al.*, 2007). The NBD domains and LRRs characterise CIITA as a member of the CATERPILLER family, which also includes CARD4/NOD1, NOD2/CARD15, CIAS1, CARD7/NALP1 and NAIP, among others (Ting and Davis, 2005). The main function of CIITA is to provide a platform for the binding of various *cis*-transcription factors such as TFIID, TFIIB, RFX, CREB and NFY, and to activate MHC class II and other genes transcription. Specifically, transcription-activation domains on the N-terminal are thought to mediate interactions with effector proteins that are implicated in promoting transcription, including components of the general transcription machinery, factors that are involved in chromatin remodelling, and other co-activators. In addition, the C-terminal two-thirds of the protein is implicated in self-association, localisation to the nucleus and recruitment to the enhanceosome (Reith *et al.*, 2001; Boss and Jensen, 2003).

2.3.1.2 DC-CIITA

The three promoters, which do not share any sequence homology and are not co-regulated, give rise to *CIITA* transcripts with three distinct first exons and the shared downstream exon (Figure 1). This leads to the production of three types of transcripts (DC-CIITA, Type III and IV CIITA) that have different 5' ends. Type IV CIITA uses the translation initiation codon in the second exon and translates into an 1106 amino acid protein. On the other hand, DC-CIITA and Type III CIITA use different initiation codons on the first exon which are both in frame with the initiation codon of Type IV CIITA. The use of these alternative

initiation codons leads to the synthesis of protein isoforms of 1207 and 1130 amino acids, respectively (LeibundGut-Landmann1 et al, 2004).

The DC-CIITA-specific N-terminal extension contains a unique sequence that shows a low similarity to CARD domain (Nickerson *et al.*, 2001). The CARD domain is a homotypic protein interaction module composed of a bundle of six alpha helices arranged in topology homologues to the death effector domain (DED). The CARD domain typically associates with other CARD-containing proteins, forming either dimers or trimers. Even though the DC-CIITA-specific N-terminus has a weak homology to the CARD, unlike other CARD-containing proteins, DC-CIITA does not interact with members of the caspase family or regulate apoptosis (Nickerson *et al.*, 2001). It has also been shown that CIITA CARD does not activate the NF-κB promoter. However, it was found that the CARD-like domain specific to DC-CIITA confers a higher transactivation activity when compared to type III CIITA without this CARD-like domain (Nickerson *et al.*, 2001).

The elucidation of the function of the CARD-like domain (unique to DC-CIITA) will be particularly important in the understanding of the function of DCs because it has been shown that CIITA expression in DCs is driven mainly by promoter I at the mRNA level. Moreover, DC-CIITA transcripts are by far the most abundant mRNA isoforms in immature bone marrow-derived DCs (Leibundgut-Landmann *et al.*, 2004). Nevertheless, there has been a lack of research on the molecular mechanisms that underlie the function of the CARD-like domain in CIITA. It has been hypothesised that the CIITA CARD-like domain may interact with a CARD of an unknown protein or proteins, possibly a

transcription factor or factors, or a protein that cooperates with the CIITA complex to enhance the transactivation of the MHC class II gene (Nickerson *et al.*, 2001). It will be interesting to find out more about this unknown protein or proteins interacting with CARD. However, the study cannot rule out the possibility that the higher transactivation activity of DC-CIITA can be attributed to more efficient nuclear translocation and increased accumulation in the nucleus. Therefore, a comprehensive study on the CARD domain is required for a fuller understanding of the function of DC-CIITA.

2.3.2 Regulation of MHC class II by CD74 at post-translational level

2.3.2.1 CD74

Antigen presentation requires the help of not only MHC class II, but also CD74. In CD74 null mice, MHC class II molecules accumulate in the ER; then antigen-presenting cells lose their ability to present exogenous antigens effectively (Viville *et al.*, 1993). The *CD74* gene encodes a type II transmembrane glycoprotein that exists in several distinct forms that arise by alternative splicing in the mice, and by both alternative splicing and alternative translation initiation in the human (O'Sullivan *et al.*, 1987). In the mouse, CD74 exists in two forms: p31 (31kDa) and p41 (41 kDa). P31 is more abundant than p41. p41 arises by splicing in exon 6b, which encodes a cysteine-rich domain of 64 amino acids into the CD74 transcript. In humans, four CD74 gene-encoded protein products, p33, p35, p41 and p43, arise by two translation initiation sites within two alternatively spliced transcripts; p33 is the predominant protein product expressed (Ceman and Sant, 1995).

CD74 is a non-polymorphic type II integral membrane protein. Murine CD74 has a short (30 amino acid) N-terminal cytoplasmic tail, followed by a single 24-amino acid transmembrane region and a 150-amino acid-long luminal domain. The N-terminal cytoplasmic tail of CD74 contains two extensively characterised dileucine-based endosomal targeting motifs (Pond *et al.*, 1995). As a result, the newly synthesised CD74 is directed into the intracellular membrane trafficking system, starting from the Golgi complex to end in the lysosome. During the journey, CD74 goes through stepwise degradation: the full-length CD74 (p31) is cleaved first into p22, then into p10. The p10 is further processed into the class II associated invariant chain peptide (CLIP), which then dissociates from MHC class II with the help of H2-M. H2-M facilitates the exchange of CLIP for the antigenic peptides (Denzin and Cresswell, 1995; Hsing and Rudensky, 2005). The peptide-loaded MHC class II molecules then leave this compartment and are expressed on the cell surface and surveyed by $CD4^+$ T cells.

In DCs, the function of CD74 is far from being just a chaperone for the MHC class II. It also regulates other proteins involving in antigen presentation pathway. For example, the CD74 isoform, p41, binds to the active site of cathepsin L and permits the maintenance of a pool of mature enzymes in the endosomal compartments of DCs (Fiebiger *et al.*, 2002). H2-M, which is required for efficient MHC class II antigenic peptide loading, is down-regulated in mature mouse DCs from $CD74^{-/-}$ mice (Pierre *et al.*, 2000). CD74 is also required for CDw78 expression, which is specific for MHC class II-associated with tetraspanin proteins on DCs (Kropshofer *et al.*, 2002; Poloso *et al.*, 2006). Moreover, CD74 alone is also able to regulate the immune response. For instance the Up-regulation of the CLIP peptide on mature DCs was shown to antagonise the T helper type 1

polarisation (Rohn *et al.*, 2004). In H-2^K mice, the knockdown of CD74 by siRNA was shown to increase significantly allogeneic lymphocyte proliferation, and to polarise allogeneic lymphocyte towards the Th1 response, by increasing IFN-γ and decreasing IL-4 production (Ke *et al.*, 2007). CD74 is also a receptor for migration inhibitory factor (MIF). The binding of CD74 to MIF and then to CD44 is known to activate extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway and to suppress cell apoptosis. In DCs, the MIF /CD74 pathway increases the antigen presentation capacity of DCs, and the production of IL-1β and IL-8 (Mukarami *et al.*, 2002;Leng *et al.*, 2003;Shi *et al.*, 2006).

2.3.2.2 Regulation of CD74 expression

The transcription regulation of CD74 was studied extensively in the 1990s. The regulatory mechanisms of CD74 expression share significant similarities with that of MHC class II. The transcriptions of both genes are under the control of CIITA. These genes are even required the same set of promoter elements (S box, X box and a modified Y box), although their translational/post-translational regulation pathways are different (Zhu and Jones, 1990; Tai *et al.*, 1999). The degradation of MHC class II occurs via the ubiquitination pathway whereas CD74 is degraded through stepwise proteolytses. These events involve several proteases; and some of them are yet to be identified.

The use of specific protease inhibitors and murine protease gene knockouts has contributed to the identification of key enzymes involved in the terminal stages of CD74 processing (Riese and Chapman, 2000; Villadangos and Ploegh, 2000), but the exact pathway of CD74 degradation remains unclear. The

endoprotease responsible for the initiation of CD74 proteolysis has not been identified. The initiating endoprotease has so far only been identified as a leupeptin-insensitive protease (Honey and Rudensky, 2003). Therefore, asparagine endopeptidase (AEP) was believed to be the first enzyme involved in CD74 degradation because of its leupeptin insensitive properties and its cleavage site found on CD74 (Manoury *et al.*, 2003b). More importantly, AEP has been reported to initiate the degradation of CD74 in B cells (Manoury *et al.*, 2003c). However, in a recent work using gene knockout mice, AEP was shown to be redundant because AEP-deficient mice do not exhibit significant difference from wild-type mice in terms of CD74 processing (Maehr *et al.*, 2005). Becker's study with brefeldin A (one of the membrane trafficking inhibitors) treatment has also revealed that CD74 is not a substrate of AEP but a member in the regulated intramembrane proteolysis (RIP) processed protein family (Becker-Herman *et al.*, 2005). Therefore, the endoprotease responsible for the initiation of CD74 proteolysis still remains to be identified.

Proteases involved in the downstream of CD74 degradation are also not conclusive. The use of specific protease inhibitors and gene ablation studies are two main methods to identify the proteases involved in the terminal stages of CD74 processing (Riese and Chapman, 2000; Villadangos and Ploegh, 2000). Experiments performed with human B cells treated with a cathepsin S inhibitor (Riese *et al.*, 1996), or with cathepsin S-deficient APCs (Shi *et al.*, 1999; Nakagawa *et al.*, 1999) have shown that cathepsin S is essentially involved in generating CLIP in bone marrow-derived professional APCs. However, another study on knockout mice has indicated that it is haplotype dependant. Cathepsin S is only able to cleave p22 into p10 in I-A^b but not in I-A^q haploid type cathepsin S-

deficient B cells and DCs. (Nakagawa *et al.*, 1999; Hsing and Rudensky, 2005). Even in the I-A^b mouse in which cathepsin is responsible for the degradation of CD74, the machineries of CD74 degradation are also not well defined. Pierre and Mellman have shown that immature DCs, but not mature DCs, express cystatin C in lysosomes (Pierre and Mellman, 1998; Pierre *et al.*, 2000). Presumably, cystatin C inhibits cysteine protease activity in immature DCs. This result in the accumulation of MHC class II–Lip10 complexes in lysosomes and consequently the inhibition of MHC class II–peptide trafficking to the cell surface. Moreover, during the maturation of DCs, the cystatin C protein level falls and the Lip10 degradation increases. Interestingly, the cathepsin S protein level does not change. This implies that the control of cathepsin S activity is solely regulated by cystatin C protein expression. Thus, DCs may utilise the regulation of CD74 degradation as a mechanism to control intracellular transport and the surface expression of MHC class II molecules during maturation (Pierre and Mellman, 1998). However, the little co-localisation of cystatin C with MHC class II in both immature and mature DCs contradicts this hypothesis (Villadangos *et al.*, 2001). Furthermore, the knockout of the cystatin C gene exerts no effect on antigen presentation (El-Sukkari *et al.*, 2003a). These experimental discrepancies have suggested that there might be other candidates involved in the degradation of CD74.

2.4 NO regulates antigen presentation

2.4.1 NO regulates antigen presentation

Nitric oxide (NO) has been recognised as one of the most versatile players in the immune system (Bogdan, 2001). Unlike cytokines, the interaction of NO is not restricted to a single defined receptor; rather, it can react with many other

inorganic molecules (such as oxygen, superoxide or transition metals), structures in DNA (pyrimidine bases), prosthetic groups (such as heme) or proteins (leading to the *S*-nitrosylation of thiol groups, the nitration of tyrosine residues or the disruption of metal-sulphide clusters such as zinc-finger domains or iron–sulphide complexes) (Marshall *et al.*, 2000). The high-output production of NO acts as a cytotoxic factor, inducing the apoptosis of DCs and T cells (Lu *et al.*, 1996; Aiello *et al.*, 2000). At lower concentration, NO is reported to increase the expression of CD1a CD80 HLA-DR during monocyte differentiation to immature DCs through a cyclic GMP-dependent pathway (Paolucci *et al.*, 2003; Fernandez-Ruiz *et al.*, 2004). In the antigen presentation pathway, the NO is able to inhibit the activity of cystatin C which is involved in the regulation of CD74 (Natasa, 2006). Recent studies have also shown that NO remodels MHC class II trafficking by inhibiting caspase activity (Wong *et al.*, 2004). The substrates of caspases in DCs include γ -adaptin and α-adaptin, which belong to adaptor protein-1 (AP-1) complex and AP-2 complexe, respectively. During maturation, the decrease of caspase activity accompanies the increase of the intact forms of α- and γ-adaptin. This implies that the accumulation of AP-1 and AP-2 enhances the cell surface expression of MHC class II. However, the silencing of both AP-1 and AP-2 in DCs or Hela-CIITA cells does not alter MHC class II cell surface expression (Santambrogio *et al.*, 2005). Moreover, the speed of clathrin-mediated endocytosis is unaltered in mature DCs (Delamarre *et al.*, 2005). These inconsistencies suggest that NO and caspase might interact with other antigen-presenting-related proteins besides membrane trafficking-related molecules.

The trafficking of MHC class II in DCs is multidirectional and reversible. The newly synthesised MHC class II can be directed to the *trans*-Golgi network

(TGN) or plasma membrane. MHC class II, which arrives on the plasma membrane, can even be retrieved back to early endosomes and recycled back to the plasma membrane. Therefore, besides α-adaptin and γ-adaptin, it is possible that the regulation of MHC class II trafficking requires other endosomal proteins such as syntaxins, dynamins, Vti1a and Vti1b. Furthermore, it is also critical as a "balancing act" amongst these trafficking related molecules in order to maintain a proper distribution of MHC class II. Previous studies have shown that NO regulates the expression of these proteins. Therefore, NO may be one of the key molecules regulating MHC class II trafficking and antigen presentation in DCs (Villadangos, *et al* 2005, Wong *et al*, 2006).

2.4.2 The source of NO

NO is synthesised by nitric oxide synthase (NOS). NOS catalyses a five electron oxidation of a guanidine nitrogens of $_L$-arginine to produce NO and cirtulline as by-product. N^G-monomethyl-$_L$-arginine ($_L$-NMMA) and other NG-substituted-$_L$-arginine derivatives competitively inhibit the NO synthases (David and Timothy, 2008).

There are three distinct isoforms of NOS: NOS1, NOS3 and NOS2. NOS1 is also called neuronal NOS (nNOS), which is predominantly expressed in neuronal cells, skeletal muscle cells and cardiac muscle cells (Kishimoto, *et al*, 1992, Hall, *et al*, 1996). NOS3 or endothelial NOS (eNOS) mainly expresses in endothelial cells, cardiac myocytes and blood platelets (Schuman and Madison, 1991). Both NOS1 and NOS3 are constitutively expressed. The third NOS enzyme, NOS2 or inducible NOS (iNOS) is contrasted with the other two NOS isoforms in several aspects. First, a plethora of human cells have been shown to express NOS2

including all of antigen presenting cells such as B cells, macrophages and dendritic cells. Second, the expression of NOS2 is not constitutive but is inducible by various cytokines, and microbial products, for example LPS. Third the activity of NOS2 is independent on calcium, which is critical for the activity of NOS1 and NOS3 (David and Timothy, 1998).

In DCs, the production of NO is highly dependent on NOS2 (Lu *et al.*, 1996). In immature DCs, both mRNA and protein levels of NOS2 are very low. And the amount of NO is also nearly undetectable. After DCs are stimulated to undergo maturation with IFN-γ or LPS *in vitro*, NOS2 mRNA and protein expression levels are up-regulated drastically and at the same time, NO is produced in large amount.

To fully understand the biological functions of NO in the immune system, the use of the NO-donor as an exogenous source of NO is essential. Currently, there are three groups of NO donors that are used in pre-clinical studies and clinical trials. These are sodium nitroprusside (SNP), diaseniumdiolates (NONOate) and *S*-nitrosothiols (Lu *et al.*, 1996; Miller and Megson, 2007). Compared with the other two NO donor, NONOate is more popular in most experimental settings due to the predictable nature of NO release. Its ionic forms have reproducible rates of spontaneous NO generation at a physiological pH. Thus, we have chosen to use NONOate in this study (King, 2005).

2.5 Summary and the importance of this study

In summary, CIITA, CD74 and NO are all important molecules in the antigen presentation pathway. However, several key details about these molecules remain

poorly understood. (1) CIITA is essential for the expression of MHC class II, CD74 and H2-M in several APCs. In DCs, the major form of CIITA is the DC-CIITA isoform which contains an additional CARD-like domain. The function of this domain still remains unknown. (2) CD74 directs the folding, trafficking and functioning of MHC class II by a stepwise degradation pathway. This pathway was believed to be under the control of the cystatin C and cathepsin pathway. However, the possibility of the existence of other pathways cannot be excluded due to inconsistent experimental data as has been earlier reviewed. (3) NO is also involved in the antigen presentation pathway by modifying cystatin and caspase activities. However, direct evidence of NO regulating antigen presentation in DCs is still lacking. Addressing the above gaps mentioned will provide insights into the MHC class II antigen presentation pathway of DCs, and eventually lead to a novel strategy for DC-based immunotherapy (Figure 5).

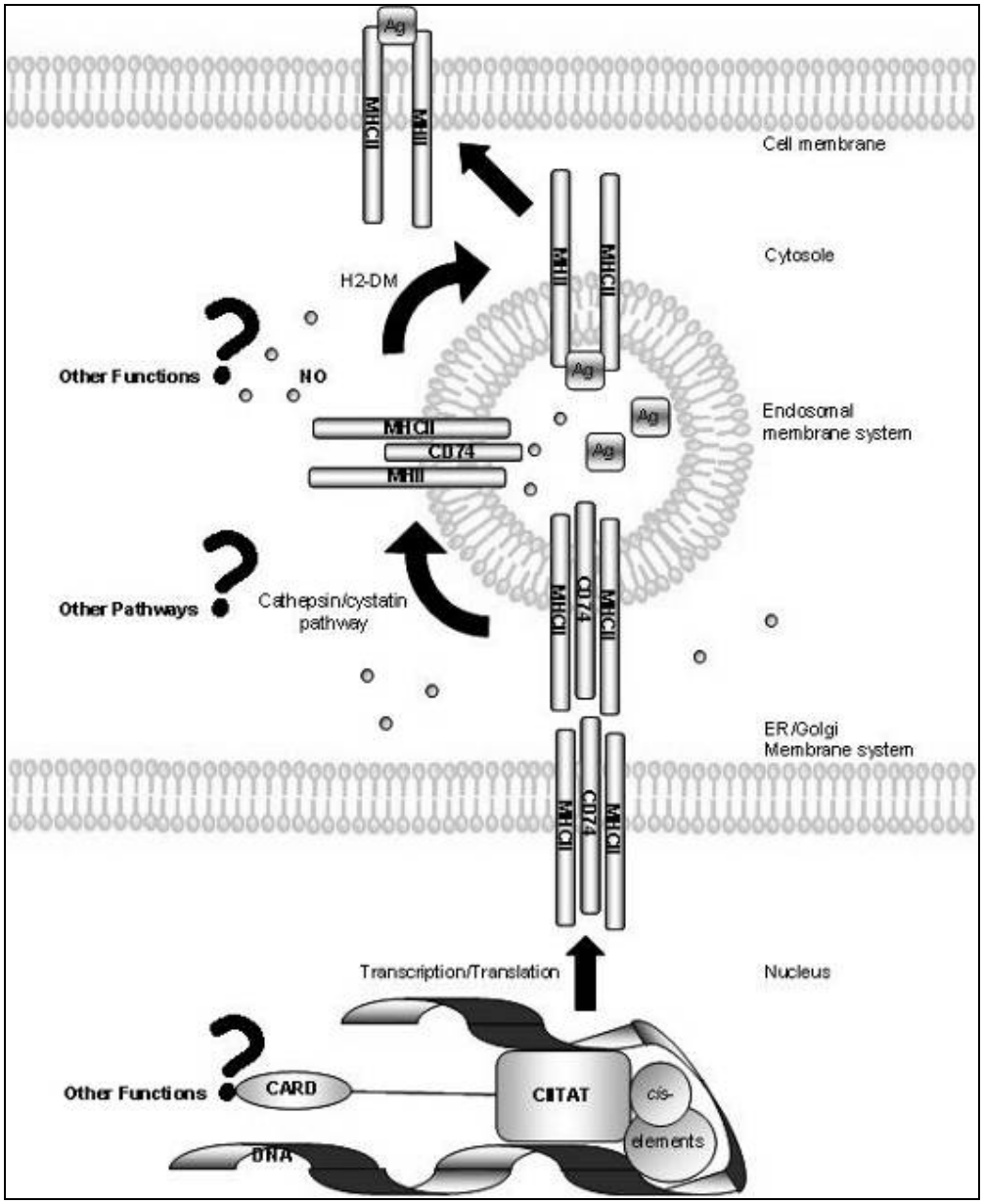


Figure 5: Summary of gaps on the studies of antigen presentation in DCs, which was investigated in this project

CHAPTER 3

A NOVEL SPLICE-ISOFORM OF CIITA REGULATES NOS AND ANTIGEN PRESENTATION IN MATURING DENDRITIC CELLS

3.1 MATERIALS AND METHODS

3.1.1 Mice

C57/BL 6J (I-A^b, I-E^b) and BALB/c (I-A^d, I-E^d) mice were purchased from centre for Animal Resources, Singapore. All protocols on mice are conducted under the Institutional Animal Care and Use Committee (IACUC), National University of Singapore.

3.1.2 Cell lines and cell culture medium

Dendritic cell line, DC2.4, was kindly provided by Dr. Kenneth Rock (University of Massachusetts Medical Centre, Worcester, USA). DC2.4 cells were generated from C57/BL 6J mouse bone marrow culture. And the cells were immortalised by transducing GM-CSF and by supertransfecting with *myc* and *raf* oncogenes. DC2.4 cells displayed dendritic morphology, and expressed the dendritic cell-specific markers CD205 as well as high levels of MHC molecules and costimulatory molecules (Shen et al. 1997).

Human epithelial carcinoma cell line, A431, was obtained from the American Type Culture Collection (ATCC) (Manassas, USA). A431 cells were established from an epidermoid carcinoma in the vulva of an 85 year old female patient, and it is a model cell line used in biomedical research. Dulbecco's Minimal Eagles medium (DMEM), Opti-MEM, fetal bovine serum (FBS), MEM non-essential amino acids and Effectene transfection reagents were purchased from Invitrogen (Carlsbad, USA). Tissue culture flasks, plates, dishes and other disposables were purchased from Nunc (Rochester, USA).

3.1.3 Antibodies and reagents

Lipopolysaccharide (LPS), *E. coli*, caspase inhibitors and chromatic caspase activity kit were purchased from Calbiochem (Darmstadt, Germany). Granulocyte/Macrophage colony stimulating factors (GM-CSF) were purchased from R&D Systems (Minneapolis, USA). Greiess reagent and Tetramethyl benzidine (TMB) were purchased from Sigma-Aldrich (St. Louis, USA). Ficoll-paque plus was purchased from GE Healthcare (Bjőrkgatan, Sweden). Other chemical reagents which are not mentioned here are all purchased from Sigma-Aldrich (St. Louis, USA).

Protein A and protein G coupled sepharose beads were purchased from Amersham Biosciences (Buckinghamshire, UK). Reduced glutathione and glutathione agarose beads were purchased from Sigma-Aldrich (St. Louis, USA).

QIAquick gel extraction Kit, Miniprep DNA Purification Kit and Maxi DNA Purification Kit were purchased from Qiagen (Hilden, Germany).

Restriction enzymes, T4 DNA ligase, *Taq* DNA polymerase, *Pfu* DNA polymerase, M-MLV reverse transcriptase, and calf intestine alkaline phosphatase (CIP) were purchased from Promega (Madison USA). 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) was purchased from Vector Laboratories (Burlingame, USA).

The primary antibodies used in this study are summarised in table 2. The various goat anti-mouse/anti-rabbit immunoglobulin conjugated to either fluorescein isothiocyanate (FITC), cyanine dyes 3 (Cy3) or rhodamine which were using in immunofluorescence staining were purchased from Jackson

Immunoresearch Laboratories (West Grove, USA). The corresponding IgG for isotype control were purchased from BD Biosciences (San Jose, USA).

The secondary antibodies, *i.e*. goat anti-mouse / anti-rabbit antibodies (HRP-conjugated) and nitrocellulose membrane were purchased from Amersham Biosciences (Buckinghamshire, UK). West Pico Supersignal substrate/enhancer was purchased from Perice Chemical (Rockford, USA).

Table 2: Antibodies used in DC-CASPIC study

Antibody	Isotype	Clone name	Format	Company
C-myc	Mouse IgG_1	9E10	--	EMD Biosciences, Inc. Calbiochem, USA
NOS2	Mouse IgG_1	2	--	BD Biosciences, San Jose, USA
NOS2	Rabbit IgG	Polyclonal	--	BD Biosciences, San Jose, USA
NOS3	Mouse IgG_1	3	--	BD Biosciences, San Jose, USA
I-A/I-E	Mouse IgG_{2b}	M5/114.15.2	--	BD Biosciences, San Jose, USA
Cytochrome *c*	Mouse IgG_{2b}	7H8	--	Santa Cruz Biotechnology Inc., Cruz, USA
β-actin	Mouse IgG_1	AC15	--	Sigma-Aldrich, Inc. USA
c-myc	Rabbit IgG	Polyclonal	--	Santa Cruz Biotechnology Inc., Cruz, USA
Caspase 1	Rabbit IgG	Polyclonal	--	Santa Cruz Biotechnology Inc., Cruz, USA
Caspase 3	Rabbit IgG	Polyclonal	--	Santa Cruz Biotechnology Inc., Cruz, USA
Caspase 11	Goat IgG	Polyclonal	--	BD Biosciences, San Jose, USA
GST	Rabbit IgG	Polyclonal	--	Santa Cruz Biotechnology Inc., Cruz, USA
CD11c	Hamster IgG_1	HL3	FITC	BD Biosciences, San Jose, USA
CD80	Mouse IgG_1	L307.4	FITC	BD Biosciences, San Jose, USA
CD86	Mouse IgG_1	FUN-1	FITC	BD Biosciences, San Jose, USA
$I\text{-}A^b$	Mouse IgG_{2a}	25-9-17	FITC	BD Biosciences, San Jose, USA

3.1.4 Culture of DCs

C57/BL 6J mice ($I\text{-}A^b$, $I\text{-}E^b$) were sacrificed by CO_2 and sterilised by soaking into 70% ethanol for one minute. After peeling away the fur by forceps,

the hand legs were cut out and put into plain DMEM and the muscles were scraped off by forceps and blades. Before filtering with 70 μM mesh nylon screen, the hematopoietic stem cells were flushed out from the resulting long bones with 21 G needle and 1 mL syringe into DMEM supplemented with 5% FBS containing 20 ng/mL of GM-CSF. Then cell number and viability were examined by hemocytometer and trypan blue stain before seeding into cell culture dishes. On day 2, 4 and 5, half of volume of old DMEM medium was replaced with same volume of fresh complete DMEM medium with 15 ng/mL of GM-CSF. The immature DCs were harvest in day 7 by flushing out with blue tips or by 5 μM EDTA. To induce cell maturation, the day five cells were further subcultured in DMEM supplemented with 1 μg/mL LPS (Sigma-Aldrich, St. Louis, USA) for 40 – 48 hours.

3.1.5 Isolation of thymocyte

BALB/c mice were sacrificed by CO_2 and sterilised by soaking into 70% ethanol for one minute. Freshly removed thymus was placed in 60 × 15 Petri dishes containing 3 mL complete DMEM. Using a circular motion, the thymus was pressed against the bottom of the Petri dish with the plunger of 5 mL syringe until mostly fibrous tissue remained. Clumps in the suspension were further dispersed by drawing up and expelling the suspension several times through a 5 mL syringe equipped with a 19 G needle. Suspension was expelled into a centrifuge tube through a 200 μM mesh nylon screen. After washing one time with complete DMEM, the cells were resuspended into 16 mL complete DMEM. Six milliliter Ficoll paque phase was layered under the cell suspension carefully followed by centrifugation for centrifuge 15 minutes at 800 *g* at room temperature.

No brake was used in centrifugation. Monocyte cells floating on top of the high-density solution were isolated by moving pipette tip over the surface of high-density layer and by drawing cells up in a 5 mL pipette. Monocytes were transferred into another tube and washed twice with complete DMEM. Then cell number and viability were examined by hemocytometer and trypan blue stain before seeding into cell culture dishes. Cells were seeded into 24-well plate with a cell density of 3×10^6 cells/well and were maintained in the DMEM medium supplemented with 50 ng/mL of IL-2.

3.1.6 Culture of cell lines

DC2.4 and A431cells were maintained in DMEM supplemented with 10% FBS, antibiotics and L-Glutamine (Invitrogen, Carlsbad, USA). The mammalian expression vectors used was pDMyc which was modified pCINeo vector with two copies of Myc tag at the 5' end. Cells were plated into plates or dishes and grown for 24 hours to a confluency of 50–60%. Lipofectamine 2000 (Invitrogen, Carlsbad, USA) and Effectene (Invitrogen, Carlsbad, USA) were used as transfection reagents for DC2.4 cells and A431 cells, respectively, according to the manufacturers' instructions. Cells were harvested for microscopic examination 20 hours after transfection. DC2.4 cells are mainly for the study of function of DCs; And A431 cells are for protein localisation studies.

3.1.7 Phagocytosis assay

The phagocytosis assay was based on the Phagocytosis Assay Kit (Vybrant, Carlsbad, USA), with 3 modifications made to the protocol: First, 100μl of K12 fluorescence *E. coli* bioparticles was mixed with 100μl of medium to provide the

cells better conditions for engulfment. Second, the phagocytosis incubation time was reduced from 3 hr to 2 hours to avoid the phagocytic activities of cells to be compared from reaching a plateau phase. Third, quenching time was doubled from 1 minute to 2 minutes to ensure extracellular particles were sufficiently quenched. Cells seeded into a 96-well ELISA plate at a concentration of 1×10^5 to 2×10^5 cells/well were allowed to adhere for 4 hr to the bottom of the well, after which the fluorescence-conjugated *E. coli* bioparticles were added and phagocytosis was allowed to proceed for 2 hr. The bioparticles were removed and Trypan Blue was added for 2 min to quench extracellular bioparticles. The Trypan Blue was then removed and the amount of bioparticles engulfed by the cells was quantitatively measured using either SpectraMax Gemini EM ELISA plate reader at 480 nm excitation and 520 nm emission or Tecan GENios ELISA plate reader at 485 nm excitation and 535 nm emission.

3.1.8 Molecular cloning

The DNA fragment to be cloned into a specific vector was either amplified by polymerase chain reaction (PCR) or restriction digested from another plasmid DNA. The sequences of the primers used for PCR normally included the sequence recognised by specific restriction enzymes selected for the cloning. Ten microgram of vectors were typically digested overnight at 37 °C by one unit of restriction enzymes and dephosphorylated by one unit of calf intestinal phosphatase for 45 minutes at 37 °C. One unit of ligation was performed using T4 DNA ligase, with the ligation mixture incubated overnight at 4 °C. Transformation was then carried out by incubating the ligation mixture with *E. coli* DH5*α* competent cells on ice for 30 minutes. Cells were then heat-shocked

for one minute in a 42 °C water bath. Lysogeny broth (LB) was then added to the cells and the cells were incubated for 45 minutes at 37 °C for recovery. The cells were then plated out onto LB plates supplemented with 100 mg/mL ampicillin and incubated at 37 ^{o}C overnight. Recombinant clones were verified by automated sequencing.

3.1.9 Reverse transcription

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, USA) according to manufacturer's instructions. Total RNA was then recovered by precipitation with isopropyl alcohol. Synthesis of cDNA from mRNA transcripts was performed using the following: 3 μg RNA, 1.5 μg oligo (dT) in a reaction volume of 25 μL. Samples were heated at 70°C for 5 minutes, placed on ice immediately and then treated with 0.5 μL RNase inhibitor, 5 μL MLV-RT buffer, 10 mM dNTP, followed by incubation at 42 °C for two minutes. After a pulse spin, 2 unit of MLV reverse transcriptase was then added to the samples and were incubated at 42 °C for 90 minutes. After the incubation, samples were heated to 70 °C for five minutes and then stored at -20 °C.

3.1.10 PCR-based deletion mutagenesis

DC-CIITA recombinant plasmids were used as templates for mutagenesis. Template-specific mutagenic primers were designed for the PCR-based deletion mutagenesis, as shown in Table 3. Briefly, a 50 μL reaction system containing 5–50 ng wild-type plasmid, 0.25 μM of sense and antisense mutagenic primers respectively, 0.2 μM dNTPs and 2.5 unit of *Pfu* DNA polymerase was subjected to 16 thermal cycles (95 °C for one minute, 55 °C for one minute, and 72 °C for six

minutes; final extension at 72 °C for 20 minutes). Fifteen microliter of the PCR products were then digested with one unit of corresponding restriction enzymes and ligated unto respective vectors followed by transformation into *E.coli* DH5α competent cells by heat shock. The mutant sequences were then verified by automated DNA sequencing.

Table 3: Primers used in DC-CASPIC study

Constructs	Primer Sequences
DC-CASPIC	Sense 5' GGAGAATTCAATGAACCACTTCCAGGCCATCCTG 3' Antisense 5' GCTCTAGAGGTCAGTGGTTAGGGGCACTTGCTTCTCTTG 3'
DC-CASPIC(1-34)	Sense 5' GGAGAATTCAATGAACCACTTCCAGGCCATCCTG 3' Antisense 5' GCTCTAGAGGTCACTCTTCTTCCAGCAGGCC 3'
DC-CASPIC(1-69)	Sense 5' GGAGAATTCAATGAACCACTTCCAGGCCATCCTG 3' Antisense 5' GCTCTAGAGGTCACAAGTCTAAGTCCCCTTTCTC 3'
DC-CASPIC(1-95)	Sense 5' GGAGAATTCAATGAACCACTTCCAGGCCATCCTG 3' Antisense 5' GCTCTAGAGGTCAAACTCCATGGTCCCTGTAGC 3'
GST-CARD	Sense 5' GCGCGGATCCGCGAACCACTTCCAGGCCATCCTG 3' Antisense 5' GCCGGAATTCCGTCAGTCCCTGTAGCTGGTG 3'

3.1.11 Automated DNA sequencing

The ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster, USA) was used for automated cycle sequencing. Recombinant plasmid DNA was first extracted from liquid bacterial culture using Miniprep DNA purification kit as described earlier. Subsequently, a reaction system of 20 μL consisting of 2μL BigDye terminators, 3.2pmol of either forward or reverse sequencing primer, 2μL of purified plasmid DNA template, and 3 μL of 5 × reaction buffer (400 mM Tris-HCl (pH 9.0), 10 mM MgCl2) was subjected to 35 thermal cycles (95 °C one minute, 58 °C one minute, and 72 °C one minute; final extension at 72 °C for five minutes). The extension products were then precipitated with 80 μL of ethanol-sodium acetate solution (consisting of 3 μL of 3 M sodium acetate, pH 4.6, 62.5 μL of non-denatured 95% (v/v) ethanol, and 14.5

μL of deionised water) for 15 minutes at room temperature. After centrifugation at 13,000 rpm for 20 minutes, the DNA pellet was wash twice with 70% (v/v) ethanol, with an incubation time of 5–15 minutes each time followed by centrifugation at 13,000 rpm for 10 minutes. The DNA pellet was allowed to dry and was resuspended in 170 μL of Hi-Di Formamide. The resuspended DNA was heated at 95 ºC for two minutes to denature the DNA strands and was then immediately placed on ice. Ten microliter of the DNA was dispensed into a 96-well microtiter place and sent to ABI PRISM® 3100 DNA Sequencer (Applied Biosystems, Foster, USA) for automated DNA sequencing. The sequencing results were viewed and analysed by the software Vector NTI (Invitrogen, Carlsbad, USA).

3.1.12 Large-scale production of GST-tagged recombinant protein

Glutathione *S*-transferase (GST)-tagged DNA constructs (in pGEX/KG vectors, Pharmacia) were transformed into *E.coli* BL21 competent (DE3) cells. Two hundred millilitre of bacterial culture in LB medium supplemented with 100 mg/mL ampicillin was grown overnight with shaking at 37 °C. On the next day, 600 mL fresh medium was added to the culture and cells were allowed to grow for another 2–3 hours until OD_{600nm} reached 0.6. Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM. The culture was then allowed to grow for another 3–4 hours before harvesting by centrifugation for 5–10 minutes at 2,500 *g*. The pellets were resuspended in the appropriate volume of PBS and phenylmethylsulfonyl fluoride (PMSF) (a general protease inhibitor). Sonication was carried out at power setting of 7 (Misonix, Farmingdale, USA), with 4 pulses lasting 10 sec each with 15 sec

rest period between pulses. The lysed cells were then centrifuged at 20,000 *g* for 30 minutes, and supernatant was collected. Glutathione agarose beads were then added to the supernatant and binding was carried out at 4 °C with constant mixing on a roller for two hours or overnight. The beads with bound protein were then transferred into a disposable chromatographic column. After extensive washing of the beads, the GST-tagged protein was eluted with glutathione (20 mM in 0.1% (v/v) Triton-X100, 50 mM Tris in PBS). The eluted fractions were analysed for the presence of GST-tagged protein by SDS-PAGE and coomassie blue staining. Fractions containing GST- tagged protein were pooled, and dialysed in cold PBS before they were aliquoted and kept frozen at -20 °C.

3.1.13 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were cast with the Bio-Rad miniprotein III gel casting system. The resolving gel contained different percentages of acrylamide/bisacylamide mixture (30% with a ratio of 29:1) in 0.375 M Tris-Cl, pH 8.8 and 0.1% (w/v) SDS. The stacking gel contained 4% (v/v) acrylamide/bisacylamide in 0.125 M Tris-Cl, pH 6.8 and 0.1% (w/v) SDS. Polymerisation was induced by the addition of ammonium persulfate (APS) and N,N,N,N,-Tetramethyl-Ethylenediamine (TEMED). Protein samples were dissolved in SDS sample buffer (50 mM Tris-Cl, pH 6.8, 2% (w/v) SDS, 100 mM dithiothreitol (DTT), 10% (v/v) glycerol and 0.1% (v/v) bromophenol blue) and loaded onto the gel. Gel electrophoresis was typically carried out in Tris-glycine buffer (0.3% (w/v) Tris-base, 1.4% w/v glycine and 0.1% (w/v) SDS) at a constant voltage of 100 V.

3.1.14 Western blot analysis

The proteins to be identified were subjected to SDS-PAGE separation and the separated proteins were then transferred onto nitrocellulose membrane. The transfer was carried out in transfer buffer (0.3% (w/v) Tris-base and 1.4% (w/v) glycine) with a Bio-Rad wet transfer apparatus (Bio-Rad Kaboratories, Miami USA) at constant voltage of 100 V for one hour. The membrane was then blocked in 5% (w/v) skim milk followed by incubations with specific primary antibodies and appropriate secondary antibodies conjugated to horse radish peroxidase (HRP) diluted in 5% (w/v) skim milk. Membranes were washed three times with PBS + 0.05% (v/v) Tween 20 after each antibody incubation steps. Immunoreactive signals were visualised using a chemiluminescent substrate (West Pico supersignal kit, Pierce, Rockford USA) and X-ray films, which were developed using a Kodak X-ray film processor (Rochester, USA).

3.1.15 GST pull down

GST pull down of tissue lysates was used either to screen for novel interacting proteins for the GST-CARD (large-scale) or to perform interaction analysis (small-scale). In a typical large-scale GST pull down experiment, 50–100 mg of tissue lysate in 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM DTT, 1% (v/v) Triton X-100, and a cocktail of protease inhibitors was incubated with 50–100 μg of GST protein (used as negative control) or GST-tagged protein (protein of interest)-bound glutathione sepharose beads. The incubation was conducted at 4 °C with rolling for two hours or overnight. Beads were then washed twice with extraction buffer containing 0.5% (v/v) Triton X-100, twice with extraction buffer containing 0.1% (v/v) Triton X-100, and once with

extraction buffer without Triton X-100. Bound proteins were then eluted with SDS sample buffer and subjected to SDS-PAGE analyses.

In a typical analytical GST pull down experiment, 2 mg of protein lysate (with Triton-X100 concentration diluted to 0.2% (v/v) with PBS) was incubated with 10–20 μg of GST protein (used as negative control) or GST-tagged protein (protein of interest)-bound glutathione sepharose beads. Elution and SDS-PAGE separation were performed as described for the large-scale GST pull down above. Separated proteins were then subjected to Western blot analysis.

3.1.16 Immunoprecipitation

Proteins were extracted from cells and incubated on ice for one hour in cold lysis buffer (20 mM Tris [pH 8.0], 10 mM EDTA, 100 mM NaCl, 1 mM DTT, 1% (v/v) Brij 98, a polyoxyethylene ether followed by centrifugation at 13,000 rpm for 15 minutes at 4 ^{o}C. Protein exacts (1 mg) were incubated overnight with 5 μg of the corresponding antibodies bound to protein G Sepharose beads (Pharmacia) in lysis buffer plus 1% (w/v) BSA and 10% (v/v) FBS at 4 ^{o}C. Beads were then washed three times in Buffer A (20 mM HEPES, [pH 7.2], 100 mM KCl, 1 mM DTT, 10 mM EDTA, 0.2 mM ATP, 0.5% (v/v) Brij98) and three times in Buffer B (identical to Buffer A except without Brij98) before being resuspended in SDS sample buffer. Immunoprecipitated proteins and 5% of total the supernatant were separated on SDS-PAGE and analysed by Western blot.

3.1.17 Isolation of mitochondria

Cells were harvested with PBS by using cell scraper. The supernatant was resuspended in 3 mL of ice-cold IB_c buffer (10 mM Tris 1 mM EGTA 200 mM

sucrose, 10 mM MOPS pH 7.4) after washing one time with PBS. The cells were homogenised with 30–40 strokes using a Teflon pestle operated at 1,600 r.p.m. The homogenate was then transferred to a 50 mL polypropylene Falcon tube and centrifuged at 600 *g* for ten minutes at 4 °C. The supernatant was collected and transfered to a glass centrifuge tube followed by centrifugation at 7,000 *g* for ten minutes at 4 °C. The supernatant was then discarded and the pellet was washed with 200 μL of ice-cold IB_c buffer. The pellet was resuspended in 200 μL of ice-cold IB_c buffer and transfered the suspension to a 1.5 mL microfuge tube followed by centrifugation at 7,000 *g* for ten minutes at 4 °C. The pellet containing mitochondria was collected, resuspended in IB_c buffer, and kept on ice.

3.1.18 Methanol fixation and immunofluorescence staining

Cells grown on coverglasses were fixed in pre-chilled methanol for four minutes at -20 ^{o}C then transferred to room temperature for two minutes. The coverglasses were then washed (with rocking for 2–3 minutes) four times with PBS. The coverglasses were then incubated with primary antibody followed by secondary antibody for about one hour each at room temperature. Non-specific binding was removed by washing (with rocking for 2–3 minutes) three times with PBS. The coverglasses were then incubated with primary antibody followed by secondary antibody (conjugated to fluorophore FITC or Texas-Red) diluted in staining buffer (5% (w/v) BSA, 2 mM EDTA, 10% (w/v) goat serum in PBS). Non-specific binding was removed by washing (with rocking for 2–3 minutes) three times with PBS supplemented with 1% (w/v) saponin. The coverglasses were finally mounted onto glass slides with one drop of Vectashield mounting medium. The edges of the coverglass were sealed with nail polish and the slides

were then ready for viewing under a fluorescence microscope. The confocal images were taken using either Olympus Fluoview 500 microscope with Fluoview version 5.0 software (Tokyo, Japan).

3.1.19 Immunogold electron immunohistochemistry

DC2.4 cells grown on Φ 100 mm dishes were fixed with 4% (v/v) paraformaldehyde for three hours at room temperature. The cells were scraped out and washed twice with PBS. Then the cells were dehydrated by going through 25%, 500%, 75%, 95% and 100% (v/v) (two changes) ethanol followed by infiltration with L.R. White embedding medium for two days. After embedding at 50 ^{o}C for 48 hours, the blocks were cut into sections with thickness of 100nm. Sections were blocked with 5% (w/v) BSA in PBS, then incubated with rabbit polyclonal anti-mouse NOS3 antibody (1:10) and mouse anti-mouse cytochrome *c* antibody (1:10) for one hour followed by incubation with a goat anti-rabbit gold (15 nm)-conjugated IgG (1:20) and goat anti-mouse gold (5 nm)-conjugated IgG (1:20) (Sigma, St. Louis, USA). Sections were contrasted with uranyl acetate, and viewed under electron microscopy EMS208 PHILIPS (Hillsboro, USA). Negative controls were carried out by omitting primary antibodies.

3.1.20 Flow cytometry analysis

Cells were harvested with PBS supplemented with 2 mM EDTA, then washed once with staining buffer (5% (w/v) BSA, 2 mM EDTA, 2 mM NaN_3 in PBS) followed by incubation for 30 minutes on ice with the FITC-conjugated antibody. For intracellular staining, cells were fixed and permeabilised with pre-chilled methanol for two minutes, then incubated with primary antibody followed

by PE conjugated anti-rabbit IgG. After washing with staining buffer, cells were fixed with 1% (w/v) paraformaldehyde and the immunophenotypic analysis was performed on a FASCAN flow cytometry (DAKO, Glostrup, Denmark).

3.1.21 Quantification of NO

NO was assayed by measuring the concentration of stable end product NO_2^-. NO_2^- production was determined by Griess reaction. Aliquots of culture supernatant (100 μL) were incubated with 100 μL of Griess reagent (Sigma, St. Louis, USA) at room temperature for ten minutes. The absorbance at 550 nm was then measured in an automated plate reader. NO concentration was determined with reference to a $NaNO_2$ standard curve.

3.1.22 Caspase activity assay

Ten million cells were lysed with 1mL lysis buffer (50 mM HEPES, 5 mM DTT, 0.1 mM EDTA, 0.1% (v/v) CHAPS, pH 7.4) for four minutes at 4 ^oC. Twenty microgram of protein was incubated at 37 °C in a buffer containing 25 mM HEPES (pH 7.5), 10% (w/v) sucrose, 0.1% (v/v) CHAPS and 10 mM DTT, with the respective colourimetric substrates [caspase 1 substrate VI (Z-YVAD-*p*NA), caspase 2 substrate I (Z-VDVAD-*p*NA), caspase 3 substrate IV (Ac-DEVD- *p*NA), caspase-4 substrate II (Ac-LEVD-*p*NA)] (all from Calbiochem, Darmstadt, Germany), in a 96-well, flat-bottom microtiterplate. The cleavage of the substrates was quantified by spectrophotometric detection of free *p*NA (λ = 400 nm) after cleavage from the caspase peptide substrates after two hours.

3.1.23 Statistics

Two tail-student's *t*-test was used for statistical analyses.

3.2 RESULTS

3.2.1 Characterisation of cultured DCs

Bone marrow-derived cells from C57BL/6J mice were cultured in 10mm diameter dishes for five days in complete DMEM medium with 20 ng/mL GM-CSF, and the resulting cells were flushed out from dishes, and then examined by flow cytometry with FITC-conjugated CD11c and MHC class II antibodies. The results showed that 79.34% of the cells were CD11c positive (Figure 6A, C), whereas only 18.56% of the cells were MHC class II positive (Figure 6B). A low cell surface MHC class II expression is expected in immature DCs. Moreover, the size and morphology of these cells under immunofluorescence microscopy are also similar to typical immature DC phenotypes which exhibit smooth edges and fewer dendrites compared with mature DCs (Figure 6C, 7B). These results indicate that the majority of isolated cells displayed typical characteristics of immature DCs.

After induction with pathogen-related molecules such as LPS, immature DCs undergo a series of distinct changes including the formation of dendrites, an accumulation of cell surface MHC class II expression, an escalation of NO production and decrement of phagocytosis and macropinocytosis (Trombetta *et al.*, 2005). These phenotypic changes mark the maturation of DCs. To verify whether the mouse bone marrow-derived DCs are induced by LPS to become mature DCs, a series of experiments were carried out to identify any phenotypic changes. First, cellular distribution of MHC class II was studied. As shown in Figure 7A and C, before LPS induction, most MHC class II molecules were localised in the

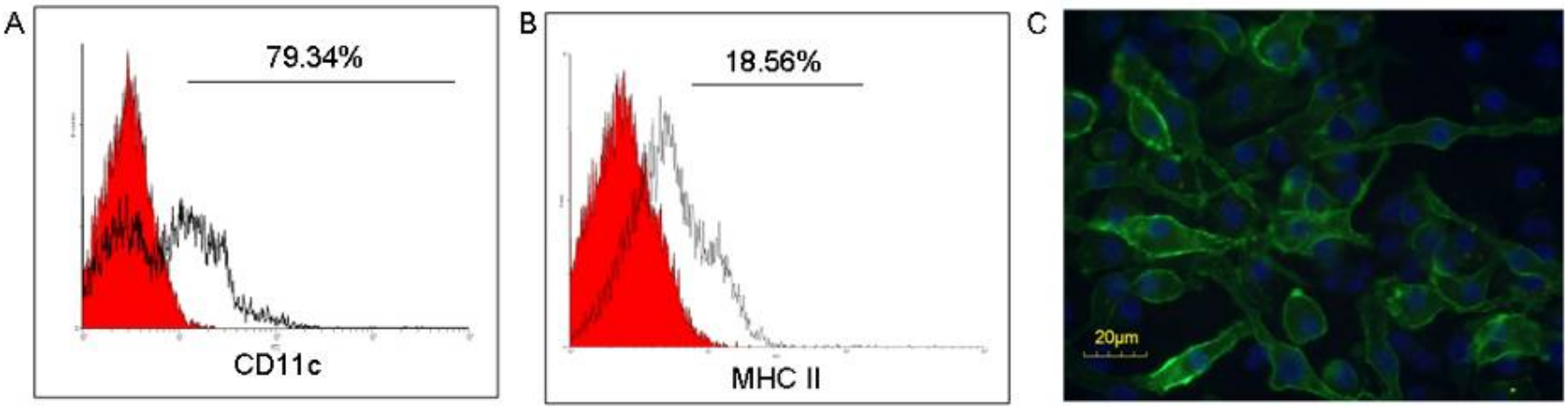


Figure 6: Culture of DCs

(A-B) Cells isolated from mouse bone marrow were cultured with 20 ng/mL GM-CSF for five days *in vitro* and stained with FITC-conjugated CD11c (Isotype: hamster IgG_1, Clone: HL3) (A) and MHC class II antibodies (Isotype: mouse IgG_{2a}, Clone: 25-9-17) (B). Stained DCs were analysed with flow cytometry and control staining (red histogram) was performed with the corresponding isotype (hamster IgG_1 for CD11c staining) and mouse IgG_{2b} for MHC class II staining). (C). The isolated cells were also fixed and stained with CD11c antibody (green) and DAPI (blue) and evaluated under fluorescent microscope.

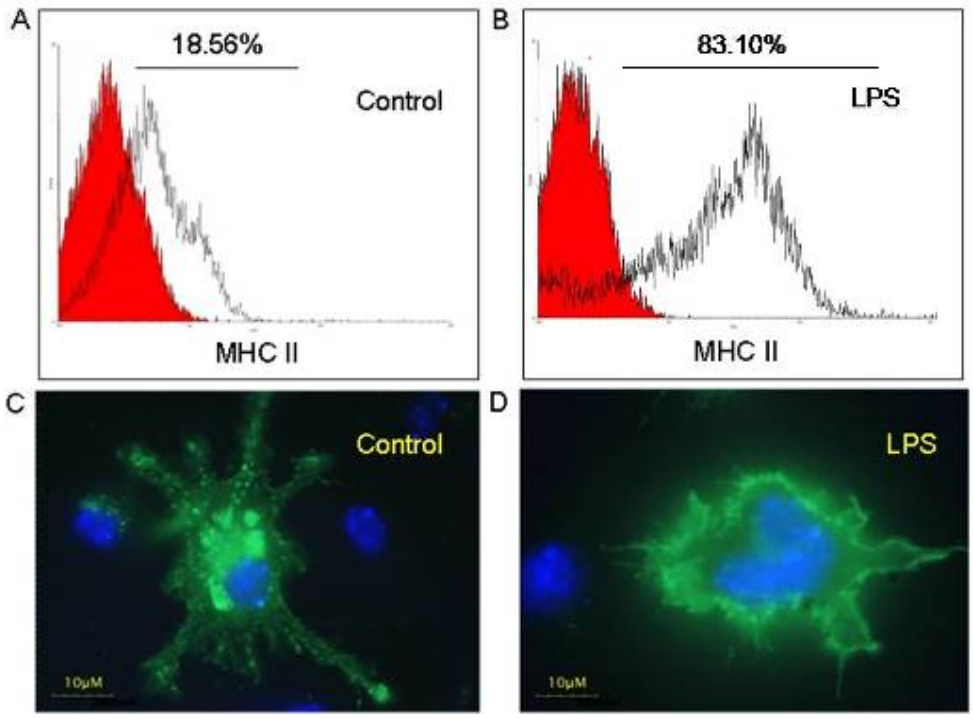


Figure 7: Redistribution of MHC class II during maturation of DCs

(A-B) Immature DCs were either untreated (control) or treated with 1 μg/mL LPS for 48 hours, and both were stained with FITC-conjugated anti-MHC class II antibodies (Isotype: mouse IgG_{2a}, Clone: 25-9-17) . Stained immature and mature DCs were analysed by flow cytometry. Control staining (red histograms) was performed with the corresponding isotype, mouse IgG_{2b} (C-D) immature and mature DCs were cultured on coverslip and stained with MHC class II-FITC antibody (green) and DAPI (blue). The cells were then evaluated under fluorescence microscope.

endosomal/lysosomal-like-structure with flow cytometry and immunofluorescence microscopy. This observation is consistent with previous studies which showed that MHC class II molecules of immature DCs are retained in lysosomes and antigen presenting compartments (Trombetta *et al.*, 2005), and after maturation, these intracellular MHC class II molecules are found on the plasma membrane. By regulating MHC class II transport and compartmentalisation, mature DCs are able to present antigens to cell surface, which are taken up and processed in endosomes and lysosomes. (Pierre and Mellman, 1998; Turley *et al.*, 2000). Accordingly, our results showed that LPS-induced bone marrow-derived DCs expressed a higher amount of cell surface MHC class II molecules (Figure 7B, D). In addition, the LPS-induced DCs exhibited more dendrites than those without LPS induction on under microscopy (Figure 7D). Next, the phagocytic activity of the isolated DCs was monitored using Vybrant Phagocytosis Assay Kit. As shown in Figure 8, the phagocytic activity decreased nine folds in LPS-induced DCs ($p < 0.01$). Lastly, NO production was monitored. NO level was increased by 8.28 folds upon LPS induction ($p < 0.01$) (Figure 9). Taken together, our results suggest that the mouse bone marrow-derived DCs were immature DCs and that LPS stimulation developed them into mature DCs.

3.2.2 Elucidation of new isoform of DC-CIITA

As an initial step to study the regulation of antigen presentation in DCs, the function of the CARD-like domain on DC-CIITA was investigated. The cDNA fragment encoding the CARD-like domain of mouse DC-CIITA was amplified by reverse transcription PCR (RT-PCR) (Figure 10) using total RNA prepared from untreated (Figure 10 Ctrl, *lane 1*) and LPS-treated DCs (Figure 10

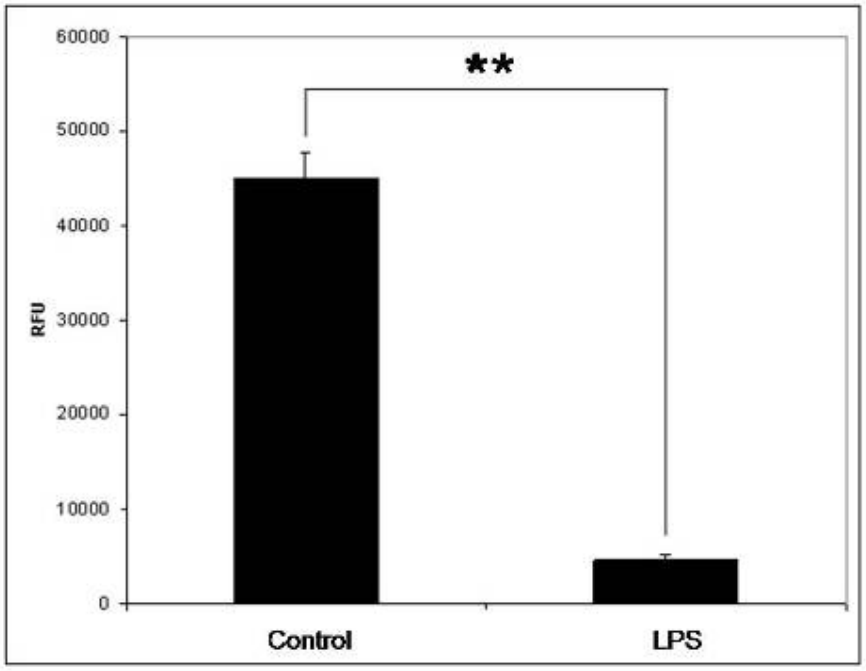


Figure 8: Inhibition of phagocytosis during maturation of DCs

Immature DCs were either untreated (Control) or treated with 1 μg/mL LPS for 48 hours and subjected to a phagocytosis assay. A fluorescent reading was taken, using a Tecan GENios ELISA plate reader (Männedorf, Switzerland). Data shown here are the mean ± *s.d.* from three independent cultures. (**, $p < 0.01$)

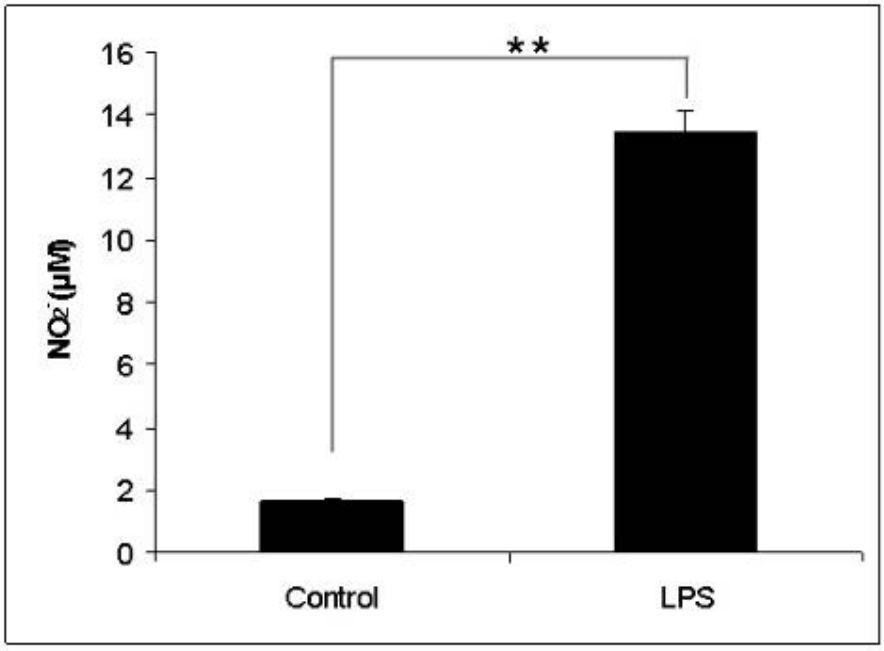


Figure 9: Increase in NO production during maturation of DCs

Immature DCs were either untreated (control) or treated with 1 μg/mL LPS for 48 hours. NO_2^- produced in the medium was qualified with Griess assay. Data shown here are the means ± *s.d.* from three independent cultures. (**, $p < 0.01$)

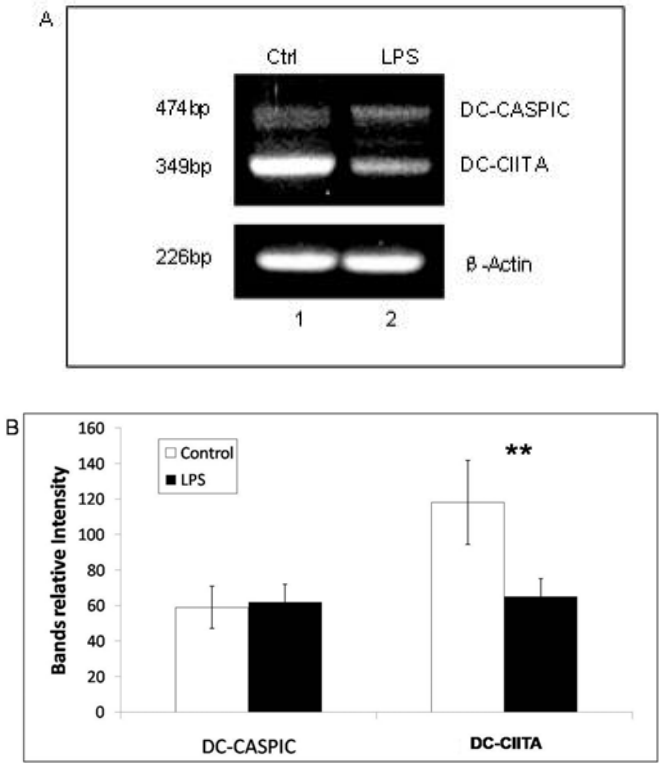


Figure 10: RT-PCR results showing a new isoform of DC-CIITA

(A) Reverse transcription-PCR was performed using immature (upper panel, *lane 1*) and mature DCs (upper panel, *lane 2)* to amplify the DNA fragment encoding the CARD-like domain of DC-CIITA. (B) The bars represent the densities of DC-CASPIC and DC-CIITA bands (normalised to *β*-actin bands). Data shown here are the mean ± *s.d.* from three independent cultures. For the convenience of presentation, the upper band is designated DC-CASPIC. (*, $p<0.05$)

LPS, *lane 2*). The primers were specific to the two flanking ends of the CARD-like domain. Interestingly, two PCR products with distinctive sizes were observed from both the treated and untreated DCs: one is approximately 350 bp while the other is approximately 450 bp (Figure 10A). The expected size of DC-CIITA is 349 bp; therefore, the lower band could likely be the CARD-like domain of DC-CIITA. The size of the upper band was about 100 bp larger than was expected. Thus, it is possible that this band was derived from other genes or other isoforms of DC-CIITA.

Furthermore consistent with previous reports (Landmann *et al.*, 2001), the mRNA of DC-CIITA is significantly down-regulated in DCs during the LPS-mediated maturation ($p < 0.05$) whereas the upper band appears unaffected by LPS treatment ($p > 0.05$) (Figure 10B).

3.2.3 Bioinformatics analysis of DC-CASPIC

The two bands from Figure 10A (*lane 1*) were excised from the agarose gel, cloned, and subjected to sequence analysis. DNA sequencing analysis revealed that both fragments encode for the CARD-like domain of DC-CIITA (Figure 11A). However, the larger fragment (474 bp) contains an additional insert of 142 nucleotides, compared to with to the smaller fragment (349 bp). This insertion has created two ORFs, ORF-1 and ORF-2. Both ORFs contain perfect Kozák sequence, which are necessary for efficient translation in eukaryotic cells. ORF-1 is longer and starts from the +194 bp position and ends at +478 bp, and it encodes for the whole CARD-like domain, with an extra stretch of 28 amino acids with no homology to any of the CIITA forms, nor to any known proteins in the SwissProt, PIR, PRF, PDB, GenBank, and RefSeq databases (data not shown).

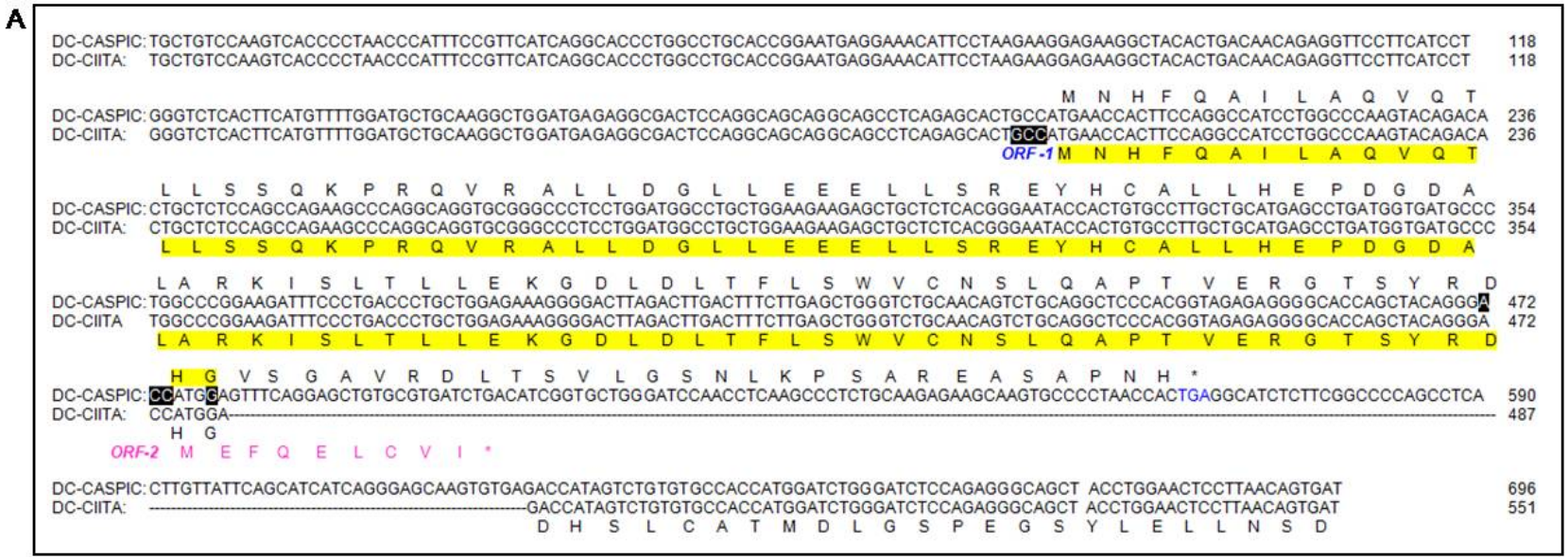
This polypeptide encoded by ORF-1 is given the name DC-expressed caspase inhibitory isoform of CIITA (DC-CASPIC). A mouse genome database search and genomic DNA sequence analysis showed that the 142-nucleotide insertion is part of intron 1 of DC-CIITA and that DC-CASPIC is a splice-isoform of DC-CIITA (Figure 11B).

ORF-2 starts from +471 bp and can be translated into a nine amino-acid-long peptide: MEFQELCYI. The NCBI BLAST result showed that it is also a novel peptide and the sequence of its first seven amino acids is very similar to one portion of mitochondrial 3-methylcrotonyl-CoA carboxylase alpha subunit (^{132}MEFAELC^{138}). Due to time constraints, this study focused only on DC-CASPIC.

3.2.4 Generation of a specific rabbit polyclonal antibody against DC-CASPIC

Although DC-CASPIC is not regulated at the mRNA level during DC maturation, it would still be interesting to investigate whether the DC-CASPIC protein level changes upon maturation by using the specific antibody against DC-CASPIC.

To generate the rabbit polyclonal antibody against the unique 28 amino acid C-terminus sequence of DC-CASPIC, the synthesised peptide, YGGAYDLTGYLGGNLKPGAREGAPNH, was injected into rabbits. The Myc-tagged DC-CASPIC construct was then transfected into A431 cells to test the specificity of the antibody. Proteins in the cell extract were separated by a 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The Myc-tagged DC-


A
DC-CASPIC: TGCTGTCCAAGTCACCCCTAACCCATTTCCGTTCATCAGGCACCCTGGCCTGCACCGGAATGAGGAAACATTCCTAAGAAGGAGAAGGCTACACTGACAACAGAGGTTCCTTCATCCT 118
DC-CIITA: TGCTGTCCAAGTCACCCCTAACCCATTTCCGTTCATCAGGCACCCTGGCCTGCACCGGAATGAGGAAACATTCCTAAGAAGGAGAAGGCTACACTGACAACAGAGGTTCCTTCATCCT 118
M N H F Q A I L A Q V Q T
DC-CASPIC: GGGTCTCACTTCATGTTTTGGATGCTGCAAGGCTGGATGAGAGGCGACTCCAGGCAGCAGGCAGCCTCAGAGCACTGCCATGAACCACTTCCAGGCCATCCTGGCCCAAGTACAGACA 236
DC-CIITA: GGGTCTCACTTCATGTTTTGGATGCTGCAAGGCTGGATGAGAGGCGACTCCAGGCAGCAGGCAGCCTCAGAGCACTGCCATGAACCACTTCCAGGCCATCCTGGCCCAAGTACAGACA 236
ORF-1 M N H F Q A I L A Q V Q T
L L S S Q K P R Q V R A L L D G L L E E E L L S R E Y H C A L L H E P D G D A
DC-CASPIC: CTGCTCTCCAGCCAGAAGCCCAGGCAGGTGCGGGCCCTCCTGGATGGCCTGCTGGAAGAAGAGCTGCTCTCACGGGAATACCACTGTGCCTTGCTGCATGAGCCTGATGGTGATGCCC 354
DC-CIITA: CTGCTCTCCAGCCAGAAGCCCAGGCAGGTGCGGGCCCTCCTGGATGGCCTGCTGGAAGAAGAGCTGCTCTCACGGGAATACCACTGTGCCTTGCTGCATGAGCCTGATGGTGATGCCC 354
L L S S Q K P R Q V R A L L D G L L E E E L L S R E Y H C A L L H E P D G D A
L A R K I S L T L L E K G D L D L T F L S W V C N S L Q A P T V E R G T S Y R D
DC-CASPIC: TGGCCCGGAAGATTTCCCTGACCCTGCTGGAGAAAGGGGACTTAGACTTGACTTTCTTGAGCTGGGTCTGCAACAGTCTGCAGGCTCCCACGGTAGAGAGGGGCACCAGCTACAGGGA 472
DC-CIITA TGGCCCGGAAGATTTCCCTGACCCTGCTGGAGAAAGGGGACTTAGACTTGACTTTCTTGAGCTGGGTCTGCAACAGTCTGCAGGCTCCCACGGTAGAGAGGGGCACCAGCTACAGGGA 472
L A R K I S L T L L E K G D L D L T F L S W V C N S L Q A P T V E R G T S Y R D
H G V S G A V R D L T S V L G S N L K P S A R E A S A P N H *
DC-CASPIC: CCATGGAGTTTCAGGAGCTGTGCGTGATCTGACATCGGTGCTGGGATCCAACCTCAAGCCCTCTGCAAGAGAAGCAAGTGCCCCTAACCACTGAGGCATCTCTTCGGCCCCAGCCTCA 590
DC-CIITA: CCATGGA---------- 487
H G
ORF-2 M E F Q E L C V I *
DC-CASPIC: CTTGTTATTCAGCATCATCAGGGAGCAAGTGTGAGACCATAGTCTGTGTGCCACCATGGATCTGGGATCTCCAGAGGGCAGCT ACCTGGAACTCCTTAACAGTGAT 696
DC-CIITA: ----------GACCATAGTCTGTGTGCCACCATGGATCTGGGATCTCCAGAGGGCAGCT ACCTGGAACTCCTTAACAGTGAT 551
D H S L C A T M D L G S P E G S Y L E L L N S D

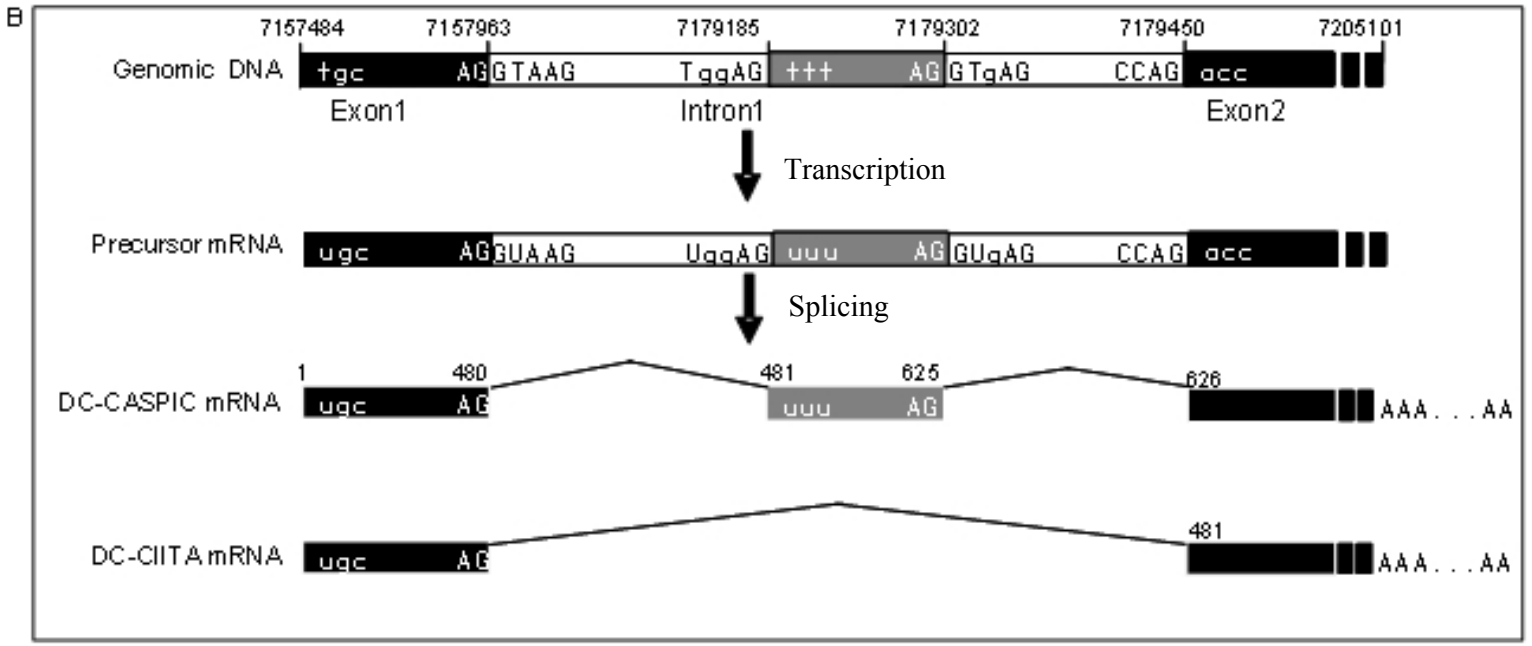


Figure 11: Bioinformatics analysis of DC-CASPIC

(A) DNA sequencing analysis revealed that the larger PCR product contains an insert of 142 nucleotides which create 2 open reading frames (ORF-1 and ORF-2). ORF-1, the longer one, encodes DC-CASPIC. The black box indicates the Kozák sequence or motif. (B) Mouse genome database search and genomic DNA sequences analysis shows that the insertion (grey box) is part of intron 1 (white boxes). Boxes in black signify CIITA exons.

CASPIC proteins were subsequently detected by a Western blotting using this polyclonal antibody. The antibody detected a 20 kDa band in cells transfected with Myc-DC-CASPIC, but not in cells transfected with a control pDMyc vector (Figure 12A, upper panel, *lane 1, 3*). Moreover, the detection of Myc-DC-CASPIC could be blocked by its immunogen, a synthetic peptide composed of the unique 28 amino acids found at the C-terminus of DC-CASPIC (Figure 12A, upper panel, *lane 4*). In DC2.4 cells, a 20 kDa band was also detected by the antibody after LPS induction, and the detection was blocked by the immunogen (Figure 12B, upper panel, *lane 2 and 4*). Taken together, the antibody showed high specificity towards both exogenous and endogenous DC-CASPIC.

To avoid the cross-reactivity with CIITA or other proteins of mouse, BLAST was carried out with antigenic peptide "YGGAYDLTGYLGGNLKPGAREGAPNH" (Expect threshold: 10). The result of BLAST showed that there are not sequences producing significant alignments with this 28-amino acid peptide in mouse. In addition, the BLAST result by using the nucleotide sequence encoding 28-amino acid peptide also showed only one unique alignment on mouse chrosome 16 genomic contig. Therefore, the algorithm analysis indicated that the possibility for this antibody to recognize other peptides was very low.

Moreover, Figure 12B also showed that the DC-CASPIC protein was indeed expressed in DCs and its level was up-regulated during LPS-induced maturation.

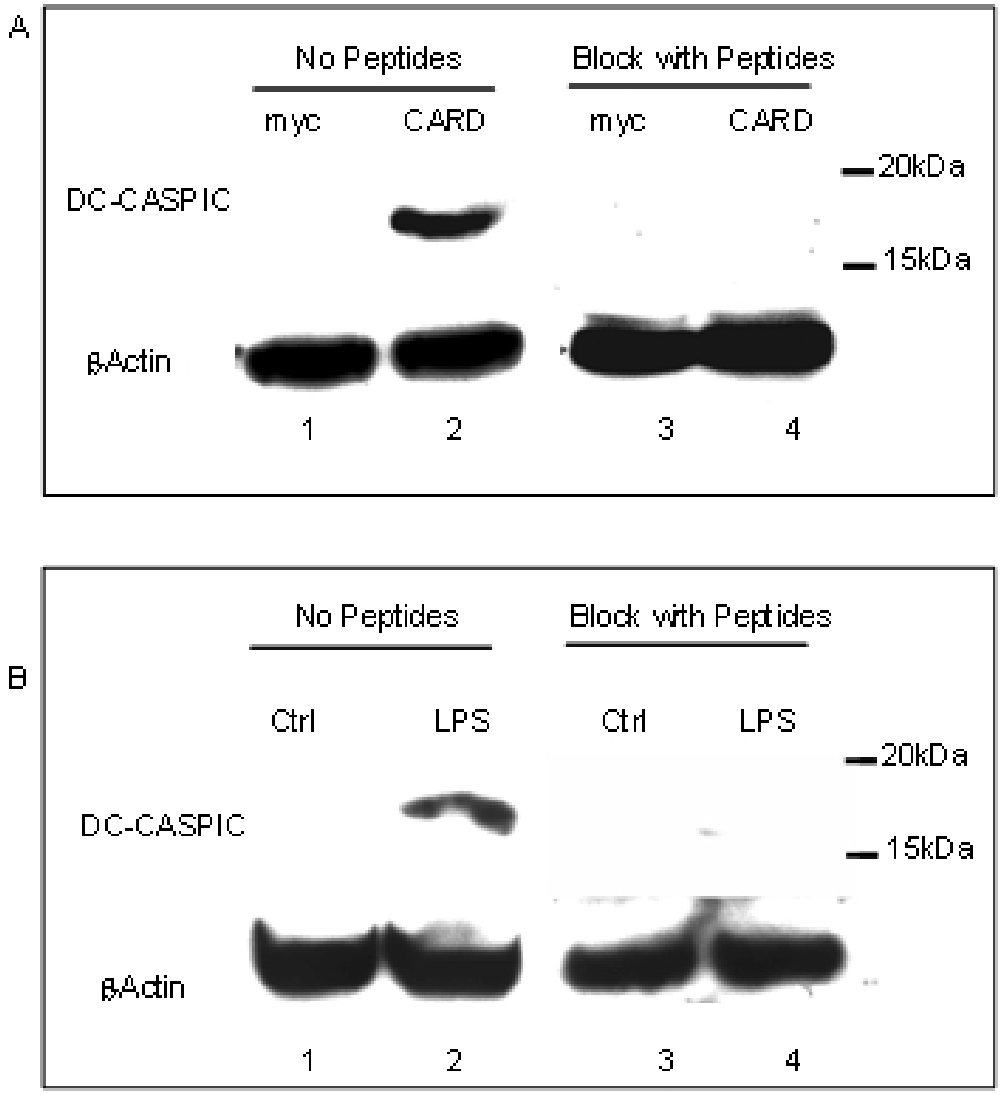


Figure 12: Verification of specificity of rabbit polyclonal antibody against DC-CASPIC

(A) The cell extracts from A431 cells, which were transfected with an empty pDmyc vector (myc) or Myc-tagged DC-CASPIC (CARD) were probed with the DC-CASPIC antibody with or without blocking of immunogenic peptide, or β-actin antibody. (B) DC 2.4 cells were treated with or without 1 μg/mL LPS for 48 hours and the cell extracts were probed with the same set of antibodies.

3.2.5 Expression profiles of DC-CASPIC

Next, we sought to analyse the expression profiles of DC-CASPIC in various APC cell lines and mouse tissues. An APC cell line survey showed that the DC-CASPIC protein was expressed in mouse DCs, dendritic cell lines DC2.4 and JAWSII and microglia N9 cell-lines, and that their expression levels are similar (Figure 13A). The tissue distribution of DC-CASPIC was also examined. Homogenates from ten different mouse tissues were tested for the presence of DC-CASPIC. Out of the ten mouse tissues tested, DC-CASPIC was detectable in six tissues, namely those of the brain, liver, kidney, small intestine and colon (Figure 13B).

3.2.6 Subcellular localisation of DC-CASPIC

DC-CASPIC in DCs has been shown to endogenously express in DCs. Next, we attempted to visualise the intracellular localisation of DC-CASPIC in DCs. Unfortunately, the DC-CASPIC antibody was not able to detect endogenous DC-CASPIC by immunofluorescence microscopy. This could likely due to the inaccessibility of the DC-CASPIC antibody to the masked epitope at the C-terminus of the naturally folded endogenous DC-CASPIC protein. Therefore, instead of detecting the endogenous DC-CASPIC in DCs, we over-expressed the Myc-tagged DC-CASPIC and detected its localisation with an antibody against Myc.

Myc-DC-CASPIC was transfected into A431 cells and the Myc-DC-CASPIC was detected with an anti-myc antibody using a confocal immunofluorescence microscope. The results showed that myc-DC-CASPIC

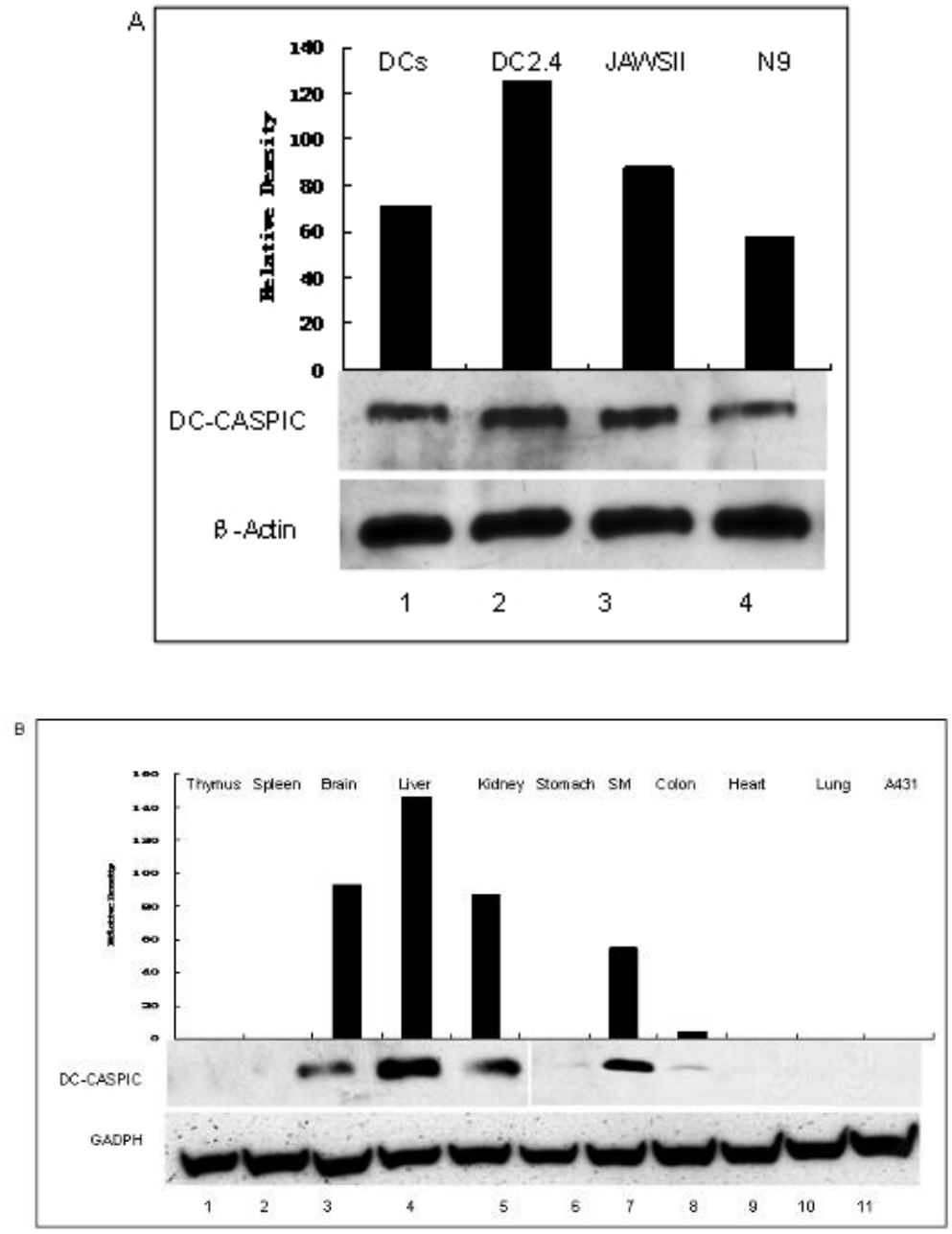


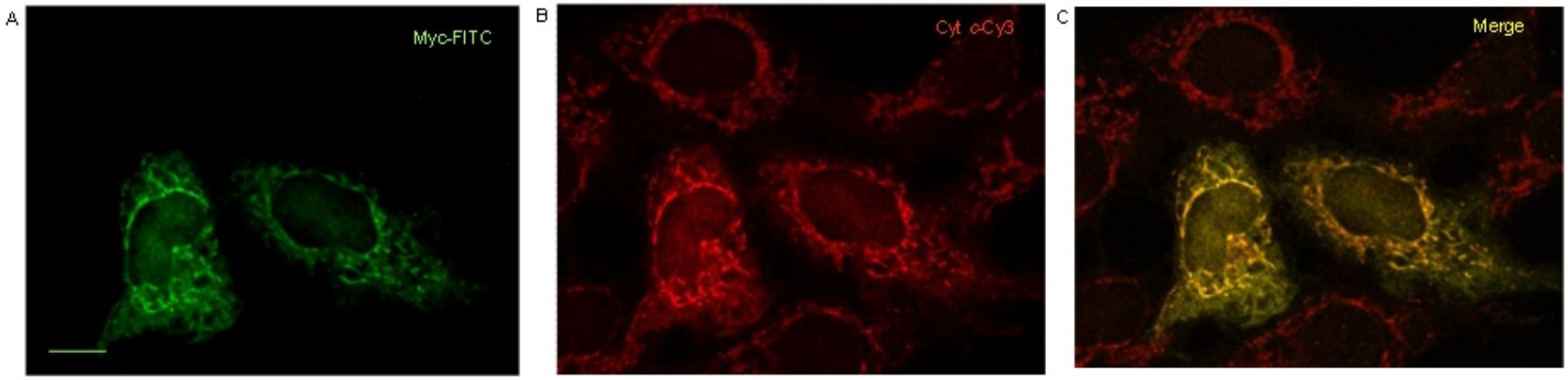
Figure 13: Expression profiles of DC-CASPIC

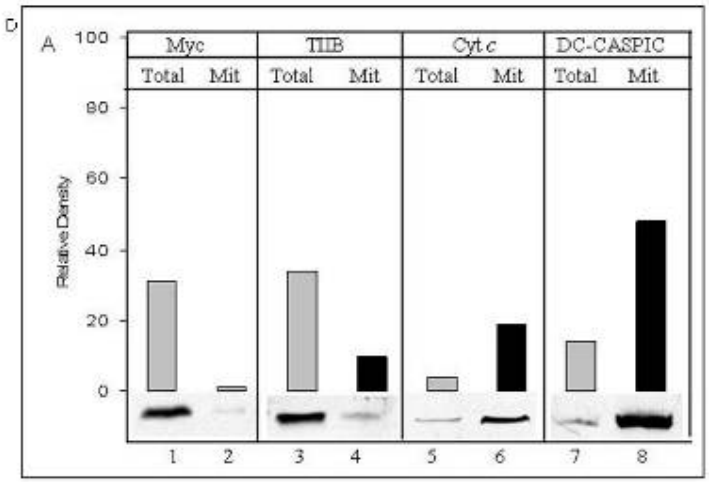
(A) Immature DCs (DCs), dendritic cells line DC2.4 and JAWSII, microglia cell lines N9, were treated with 1 μg/mL LPS and the cell extracts were probed with DC-CASPIC and β-actin antibodies. (B) Homogenates from ten different organs of a mouse (as indicated) and A431 cells were probed with DC-CASPIC and GADPH antibodies. SM, small intestine.

staining pattern displayed a typical mitochondrial-like localisation (Figure 14A). Therefore, the cells were then double labelled with anti-myc and cytochrome *c* antibody. This was then followed by staining with FITC- and cy3-conjugated secondary antibodies. The confocal immunofluorescence microscopy analysis showed that Myc-DC-CASPIC co-localised well with cytochrome *c* in the mitochondria (Figure 14A–C).

To further confirm the mitochondrial localisation of DC-CASPIC, mitochondria were isolated from DC2.4 cells according to the protocol described in *Materials and Methods* (See section 3.1.17). Then, proteins from either total cell extract (Total) or from mitochondria (Mit) were separated by SDS-PAGE followed by a Western blot analysis with specific antibodies against Myc, TFIIB, cytochrome *c*, DC-CASPIC and. As shown in Figure 14D, DC-CASPIC was enriched in the mitochondrial fraction. The Myc and TFIIB proteins served as cytosol and nucleus markers, respectively. They were detected in the total cell extract but not in the mitochondrial fraction, showing that the mitochondrial fraction was relatively pure.

The localisation of DC-CASPIC in the mitochondria was further verified by immunogold electron microscopy. Ultrathin sections of DC2.4 cells were fixed and stained with antibodies against DC-CASPIC and cytochrome *c* followed by 10nm and 5nm immunogold-conjugated secondary antibodies, respectively. Thus, DC-CASPIC and cytochrome *c* were labelled with 10 nm and 5 nm immunogold, respectively. Under the electron microscope, DC-CASPIC, as expected, co-localised with cytochrome *c* (Figure 14E) in the mitochondria. Negative staining


A
Myc-FITC
B
Cyt cCy3
C
Merge


D
A
Myc
TIIB
Cyt c
DC-CASPIC
Total
Mit
Relative Density
100
80
60
40
20
0
1
2
3
4
5
6
7
8

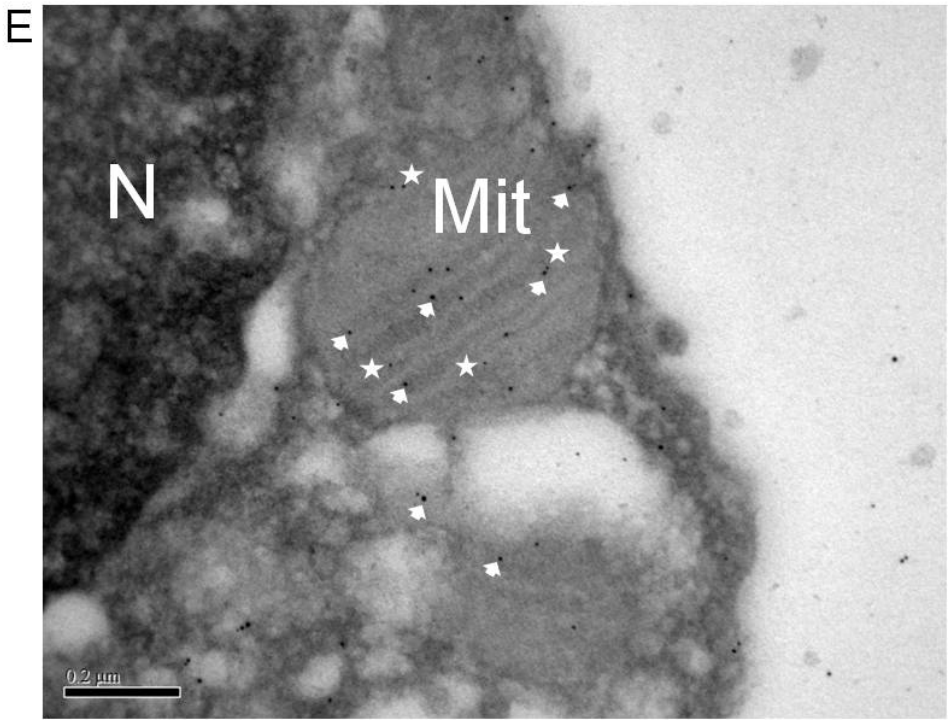


Figure 14: DC-CASPIC co-localises with cytochrome *c* in mitochondria

(A–C) A431 cells expressing Myc-DC-CASPIC were fixed and stained with antibodies against c-myc and cytochrome *c* (Cyt *c*), followed by FITC- and cy3-conjugated secondary antibodies, respectively. DC-CASPIC and cytochrome *c* were subsequently detected under a confocal microscope. (Scale bar: 10 μm) (D) Protein extract from DCs whole cells (Total) or mitochondria (Mit) was separated by SDS-PAGE and probed with myc, TFIIB, cytochrome *c* and DC-CASPIC antibodies in a Western blot. Bars represent the density of myc bands (normalised to β-actin bands). (E) Ultrthin sections of DC2.4 cells were fixed and stained with antibodies against cytochrome *c* and DC-CASPIC followed by 5nm and 10nm immunogold-conjugated secondary antibodies, respectively. DC-CASPIC (Arrow) and cytochrome *c* (Star) were subsequently detected by electron microscopy (Mit: mitochondria, N: nuclear, Scale bar: 100 nm).

in the nucleus served as a negative control. Thus, the results demonstrate that DC-CASPIC is enriched in the mitochondria.

The N-terminus of DC-CASPIC was previously identified to have a weak homology to CARD, which is characterised by six α-helices (Nickerson *et al.*, 2001). To examine whether an intact CARD-like domain is required for its mitochondrial localisation, three truncated DC-CASPIC constructs, which were made up of amino acid residues 1 to 34, residues 1 to 69 and residues 1 to 95, respectively, were generated. They were subsequently termed DC-CASPIC (1–34), DC-CASPIC (1–69) and DC-CASPIC (1–95) (Figure 15A). The three truncated constructs were cloned into a pDMyc mammalian expression vector. The recombinant protein expressed would thus be C-terminus Myc-tagged DC-CASPIC.

These constructs were respectively transfected into A431 cells and the cells were fixed and doubly labelled with anti-myc and anti-cytochrome *c* antibodies, followed by FITC- and cy3-conjugated secondary antibodies. Under the observations of immunofluorescence confocal microscopy (Figure 15B-G), Myc-DC-CASPIC (Figure 14C) and Myc-DC-CASPIC (1–95) (Figure 15B, E) perfectly co-localised with cytochrome *c* in the mitochondria. However, Myc-DC-CASPIC (1–69) co-localised with cytochrome *c* in the mitochondria, but also exhibited some extra-mitochondrial staining (Figure 15C, F). Interestingly, Myc-DC-CASPIC (1–34) mainly exhibited a cytoplasmic staining (Figure 15D, G). In comparison with DC-CASPIC (1–34) and DC-CASPIC (1–69) constructs, DC-CASPIC (1–34) has α-helices H3 and H4 deleted. This deletion of H3 and H4

A


H1a
H1b
H2
H3
H4
H5
H6
DC-CASPIC:
MNHFQAILAQVQTLLSSQKPRQVRALLDGLLEEELLSREYHCALLHEPDGDALARKISLTLLEKGDLDLTFLSWVCNSLQAPTVERGTSYRDHGVSGAVRDLTSVLGSNLKPSAREASAPNH
DC-CASPIC(1-95a.a):
MNHFQAILAQVQTLLSSQKPRQVRALLDGLLEEELLSREYHCALLHEPDGDALARKISLTLLEKGDLDLTFLSWVCNSLQAPTVERGTSYRDHGV
DC-CASPIC(1-69a.a):
MNHFQAILAQVQTLLSSQKPRQVRALLDGLLEEELLSREYHCALLHEPDGDALARKISLTLLEKGDLDL
DC-CASPIC(1-34a.a):
MNHFQAILAQVQTLLSSQKPRQVRALLDGLLEEE

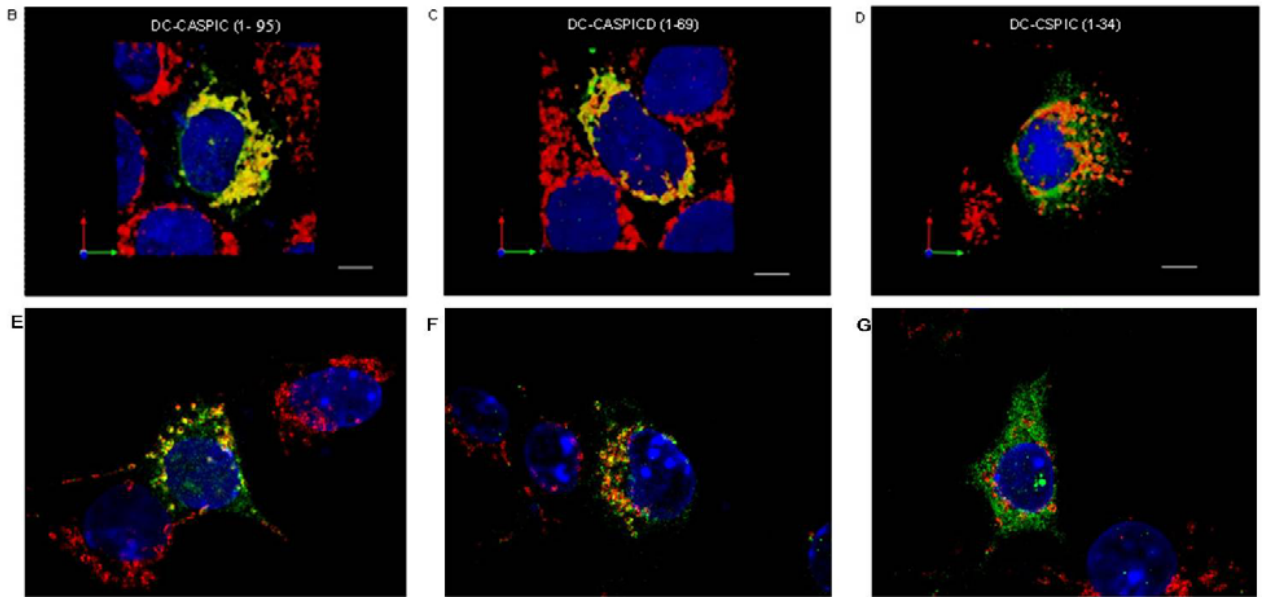


Figure 15: Localisation of different DC-CASPIC truncated constructs inA431 cells and DC2.4 cells

(A) Generation of DC-CAPSIC truncated constructs. H1a-H6 represents the six α-helices typical of the CARD domain. The final lengths of the constructs are as follows: DC-CASPIC (1–34) (1–34 residues), DC-CASPIC (1–69) (1–69 residues) and DC-CASPIC (1–95) (1–95 residues). (B–D) Different DC-CASPIC truncated constructs were transfected into A431(B-D) cells or DC2.4 (E-G). The cells were fixed and doubly labelled with antibodies against c-myc and cytochrome *c* (Cyto *c*), followed by FITC and cy3-conjugated secondary antibodies. The nucleus was stained by DAPI (blue). DC-CASPIC and cytochrome *c* were subsequently detected by confocal microscopy (Scale bar: 10 μm)

helices has fully abolished the localisation of DC-CASPIC in the mitochondria. Therefore, these two helices are essential for the mitochondrial localisation of DC-CASPIC.

3.2.7 Over-expression of DC-CASPIC enhances NO production in DCs

In view of our observation that DC-CASPIC protein expression was up-regulated in the mature DCs (Figure 12B), it is interesting to determine the possible roles of DC-CASPIC during DC maturation. The pDMyc vector and DC-CASPIC were transfected into DC2.4 and the effect of DC-CASPIC over-expression on NO synthesis (one of the key molecules produced by DCs going through maturation) was examined in the absence and presence of LPS. The culture medium from these cells was subjected to a Griess assay. The results showed that NO_2^- levels were higher in DC-CASPIC over-expressing cells for mature stages of DCs (Figure 16, $p < 0.01$). Thus, it can be concluded that DC-CASPIC boosts NO production during maturation of DCs.

To ascertain if the localisation of DC-CASPIC to the mitochondria correlates with the induction of NO synthesis, DC2.4 cells stably expressing the truncated constructs DC-CASPIC (1–34), DC-CASPIC (1–69), DC-CASPIC (1–95) or full-length DC-CASPIC (Figure 15A) were treated with LPS to induce DC maturation, then cell culture medium were then collected and assayed for NO production by measuring nitrite concentration (a breakdown product of NO). As shown in Figure 17A, NO produced in DC 2.4 cells which were transfected with full-length DC-CASPIC, DC-CASPIC (1–69) and DC-CASPIC (1–95) was approximately 3.1, 3.4 and 2.7 times higher than that of pDMyc-transfected

control cells, respectively. This result thus revealed that the deletion of the non-CARD segments on DC-CASPIC and the last two helices on the C-terminus of CARD domain deletion of H5 and H6 helices on the C-terminal CARD domain (refer to the schematic diagrams on full-length and truncated DC-CASPIC in Figure 15A did not affect the normal function of DC-CASPIC in enhancing NO production. Although DC-CASPIC (1–69) increased NO production more potently than the full-length DC-CASPIC, it should be noted that DC-CASPIC (1–69) was higher in protein expression than the full-length DC-CASPIC (Figure 17B). This might explain the lower level of NO measured for full-length DC-CASPIC than for DC-CASPIC (1–69). On the other hand, NO level produced by DC2.4 cells expressing DC-CASPIC (1–34) was similar to that of the control. Taken together, these results have thus indicated that the H3 and H4 helices are necessary for DC-CASPIC to enhance NO production in LPS-induced mature DCs.

3.2.8 Over-expression of DC-CASPIC increases NOS2 protein level

Since DC-CASPIC up-regulates NO synthesis in DCs, we next examined whether DC-CASPIC also induces the expression of NOS2, which is one of major forms of NOS in DCs. To address this issue, the pDMyc vector- and DC-CASPIC-transfected DC2.4 were either uninduced or induced with LPS. Cell lysates were separated by SDS-PAGE and analysed by a Western blot using anti-myc and anti-NOS2 antibodies. NOS2 protein expression was up-regulated in both pDMyc- and DC-CASPIC transfected DCs upon LPS induction (Figure 18A, compare *lanes 1 and 3*, and *lane 2* and *4*). Interestingly, unlike NOS3, NOS2 was

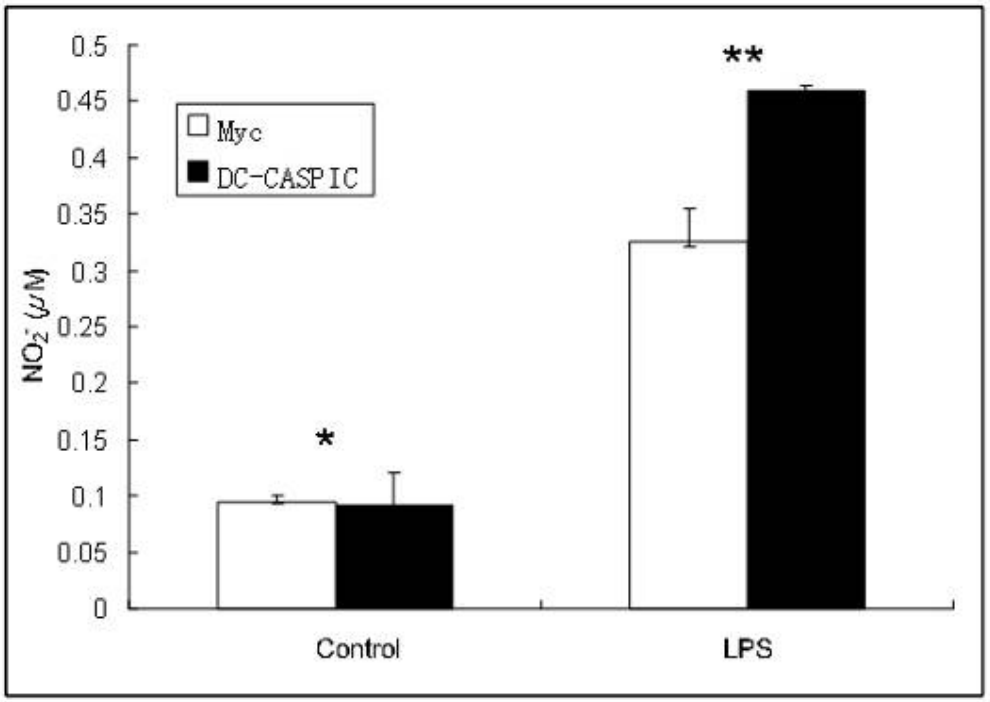


Figure 16: Over-expression of DC-CASPIC enhances NO production in DCs

DC2.4 cells were stably transfected either with a pDMyc empty vector (white bars) or DC-CASPIC (black bars), followed by no induction or induction with 1 μg/mL LPS for 48 hours. The NO production was measured by a Griess assay. (*, $p > 0.05$; **, $p < 0.01$)

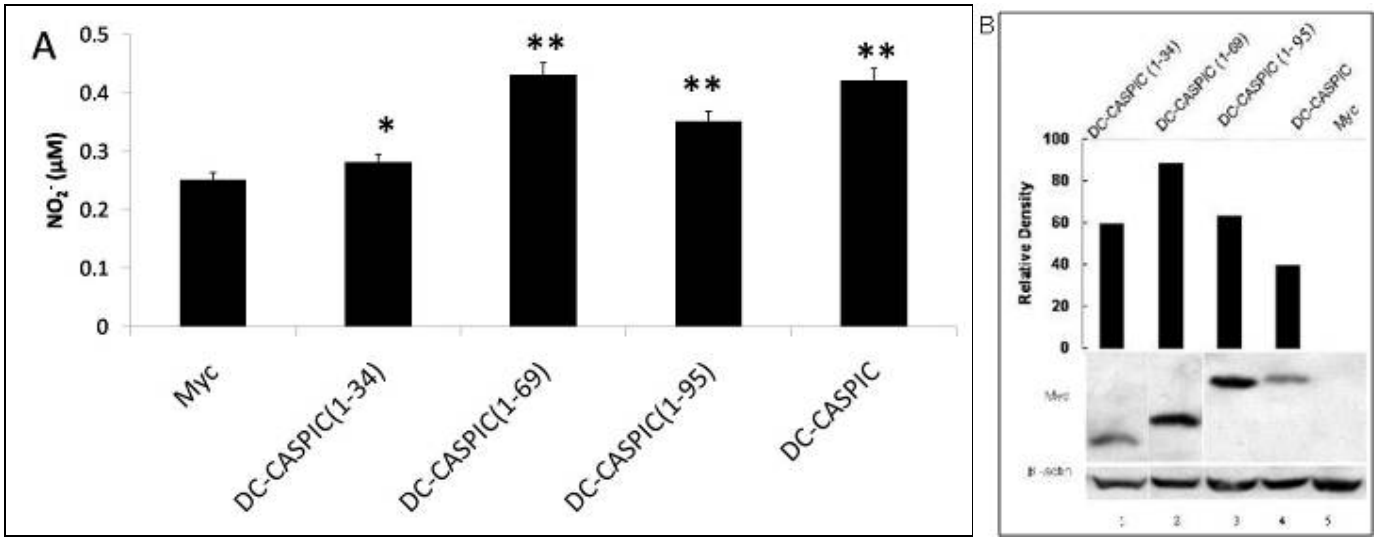


Figure 17: H3 and H4 helices are essential for DC-CASPIC to enhance NO production

(A) DC2.4 cells were stably transfected either with a pDMyc empty vector, Myc-DC-CASPIC (1–34), Myc-DC-CASPIC (1–69), Myc-DC-CASPIC (1–95) or Myc-DC-CASPIC, followed by no induction (white bar) or induction with 1 μg/mL LPS (black bar) for 48 hours. The NO production was measured by a Griess assay. Data shown here is the mean ± *s.d.* from three independent cultures. (**, $p < 0.01$; *, $p > 0.05$) (B) Cell lysates of DC2.4 expressing different constructs were subjected to a Western blot analysis using anti-myc monoclonal antibodies. β-actin was used as loading control.

up-regulated in DC2.4 over-expressing DC-CASPIC. Nevertheless, it was noteworthy that the DC-CASPIC-construct transfected cells consistently exhibited a higher NOS2 protein level compared to the pDMyc vector transfected cells, both in the immature and mature DCs. These results indicate that DC-CASPIC over-expression up-regulates the NOS2 protein level.

To determine whether the regulation of NOS2 expression by DC-CASPIC is at the transcriptional or translational level, NOS2 mRNA level was examined by an RT-PCR analysis. As shown in Figure 18B, the over-expression of DC-CASPIC did not significantly elevate the NOS2 mRNA level at either the immature or mature stages of DCs (compare *lane 1* and *2* for immature DCs, *lane 3* and *4* for mature DCs). Thus, the regulation of the NOS2 protein expression by DC-CASPIC is at a translational level, but not at a transcriptional level.

3.2.9 NOS2 is a substrate for caspases and DC-CASPIC inhibits caspase activity

In view of our observation that the DC-CASPIC-induced up-regulation of NOS2 protein expression was not regulated at the mRNA level, it is logical to speculate that NOS2 could be a substrate for proteases in the cytoplasm. An analysis of the amino acid sequence of NOS2 revealed at least two conserved potential caspase recognition/cleavage sites: ^{847}DETD^{850} for caspase 3 and ^{14}YQSD^{17} for caspase 1 (Luschen *et al.*, 1998). The ^{14}YQSD^{17} sequence is known to be essential for NOS2-NOS2 homodimers formation. Such a homodimer formation mediates NO synthesis by NOS2 (Ratovitski *et al.*, 1999). Thus, if a ^{14}YQSD^{17} sequence is cleaved by caspases, one would expect the NOS2-NOS2 homodimer formation and its activities to be disrupted. In contrast, the ^{847}DETD^{850}

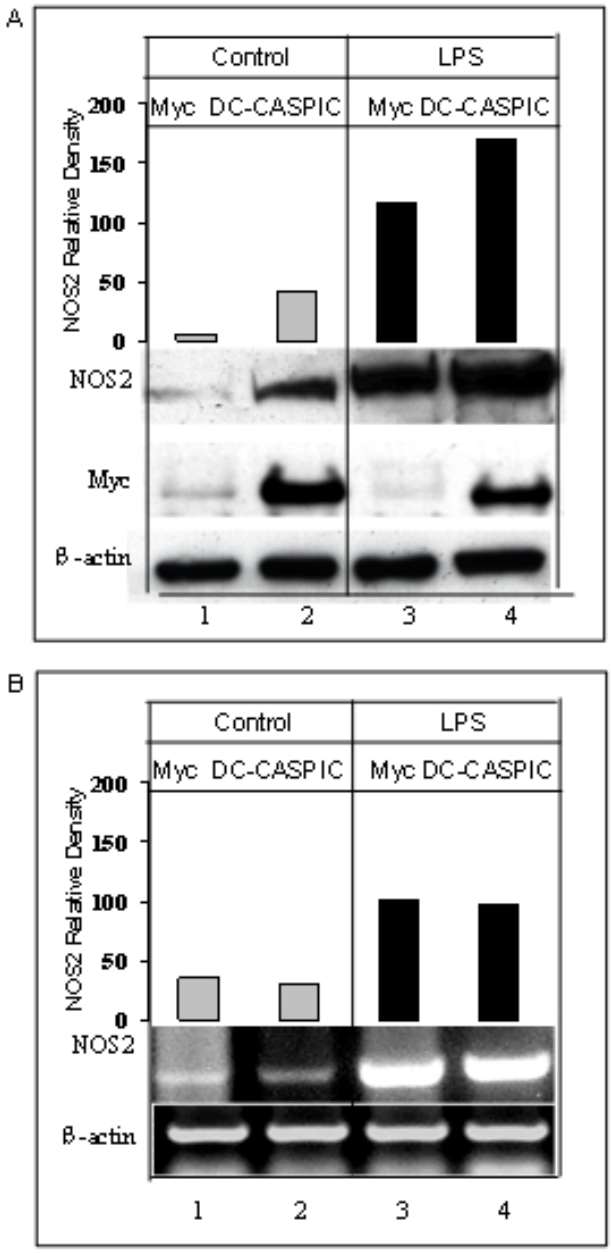


Figure 18: Over-expression of DC-CASPIC increases NOS2 expression at translational level but at not transcriptional level

(A) The pDMyc vector and Myc-DC-CASPIC construct were transfected into DC2.4 cells followed by induction with or without LPS. The cells were lysed and subjected to a Western blot analysis with the use of myc, NOS2 and *β*-actin antibodies. The bars represent the densities of NOS2 bands in the Western blot analysis (normalises to the *β*-actin bands). (B) The mRNA was isolated from the same set of cells and performed an RT-PCR analysis for the mRNA level of NOS2 and *β*-actin.

cleavage site has not been extensively studied. Nevertheless, these findings indicate that caspases may be protease candidates involved in the degradation of NOS2. To test this hypothesis, DCs were treated with either IFN-γ or a caspase inhibitor (CI) and subjected to a Western blot analysis for their NOS2 protein level. Treatment with IFN-γ served as a positive control, because IFN-γ is known to induce NOS2 gene expression. The Western blot analysis results showed that the NOS2 proteins increased upon treatment with the caspase inhibitor (Figure 19). This result implies that NOS2 is a substrate for caspases.

If NOS2 is a substrate for caspases and DC-CASPIC protects it from proteolytic degradation, DC-CASPIC may thus inhibit caspase activities. To address this possibility, a total cell extract from immature DCs was pre-incubated with 1 μg of GST-CARD recombinant fusion protein followed by incubation with various caspase substrate peptides. The changes in different caspase activities were then analysed according to the protocol described in *Materials and Methods* (See section 3.1.22). Figure 20 shows the relative activities of various caspases with their respective controls (total cell extract without pre-incubation with GST-CARD). It was observed that pre-incubation with GST-CARD significantly suppressed the cleavage of *p*-YVAD ($p < 0.01$) and *p*-DEVD peptides ($p < 0.01$). *p*-YVAD and *p*-DEVD are substrates of caspase 1 and caspase 3, respectively. However, there were no obvious changes in the cleavage of the *p*-IETD peptide, which is a substrate of caspase 8 ($p > 0.05$) (Figure 20). Therefore, DC-CASPIC could inhibit caspase 1 and caspase 3 activities, but not caspase 8 activities.

Taken together, our results suggest that DC-CASPIC protects NOS2 from protein degradation by inhibiting caspase 1 and caspase 3 activities.

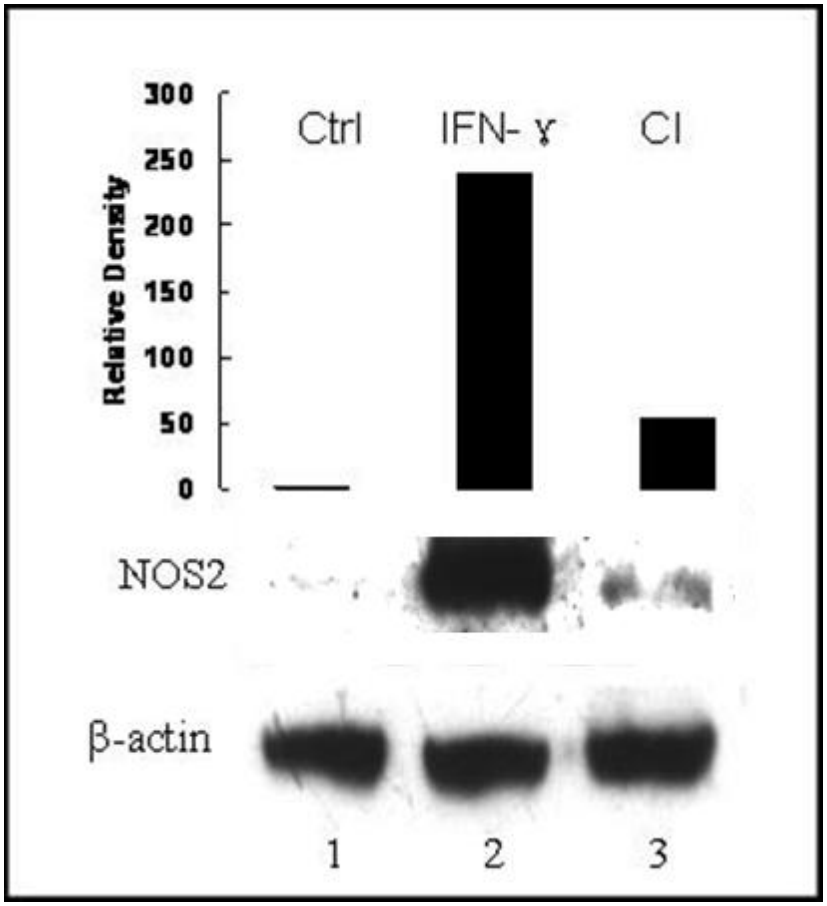


Figure 19: Caspase inhibitor causes an increase in NOS2 protein in DCs

DC2.4 cells were untreated (*lane 1*), treated with 10mg/mL IFN-*γ* for 48 hours (*lane 2*), or treated with 200 nM caspase inhibitor (CI) for four hours (*lane 3*). The cells were then lysed and subjected to a Western blot analysis, using NOS2 and *β*-actin antibodies.

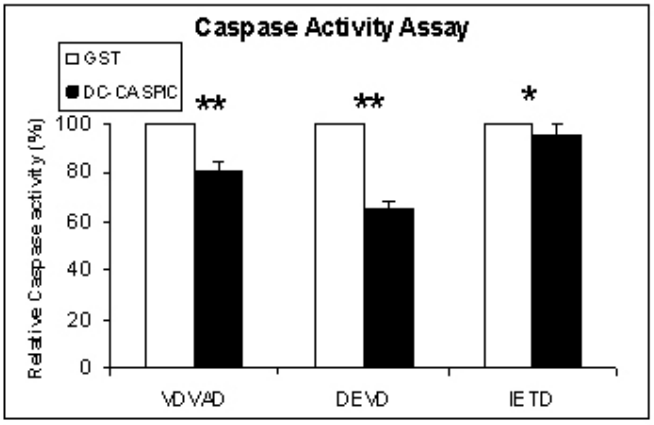


Figure 20: DC-CASPIC inhibits caspase activity *in vitro*

Cell extract (300 μg) from DCs was incubated with 1μg of GST or a GST-DC-CASPIC protein, together with caspase 1 substrate (*p*-YVAD), caspase 3 substrate (*p*-DEVD), or caspase 8 substrate (*p*-IETD), respectively, for four hours, then the caspase activities were measured. Caspase activity in the control, which was incubated with GST protein, was set as 100% percent. (**, $p < 0.01$; *, $p > 0.05$)

3.2.10 DC-CASPIC interacts with caspase 1 and caspase 3

Since DC-CASPIC inhibits caspase activity in DCs, there is a possibility that DC-CASPIC interacts with caspases through its CARD-like domain. For many CARD domain-containing proteins, their biological functions are mediated by interacting with other CARD-containing proteins through CARD-CARD interaction (Hofmann *et al.*, 1997). Thus, we hypothesised that DC-CASPIC inhibits caspase activities through CARD-CARD domain interaction as well. CARD domain at the N-terminus of DC-CASPIC was cloned into a pGEX-KG vector and expressed as a GST-tagged CARD domain (GST-CARD). The GST-CARD was then used as bait to pull down its potential interacting partners from the total DC cell extract. The GST-CARD successfully pulled down the full-length caspase 1 which contains a CARD domain and the small unit of active caspase 3 (p17), which has no CARD domain (Figure 21). However, the pull down experiments did not detect any interaction of GST-CARD with either caspase 2 or 4. Thus, our results suggest that DC-CASPIC interacts with caspase 1 and caspase 3, thereby protecting NOS2 from being degraded, by suppressing the proteolytic activities of caspases 1 and 3 (Figure 21).

3.2.11 NOS3 localises to mitochondria

Our results show that DC-CASPIC localises to the mitochondria and DC-CASPIC over-expression in DCs protects NOS2 from degradation (presumably by caspases) (Figure 14 and 18). In order to protect NOS2 from caspase-dependent degradation, it is important for DC-CASPIC and NOS2 to localise in proximity to each other. To address this question, DCs were fixed and incubated with antibodies against NOS2 and cytochrome *c*, followed by FITC and cy3-conjugated

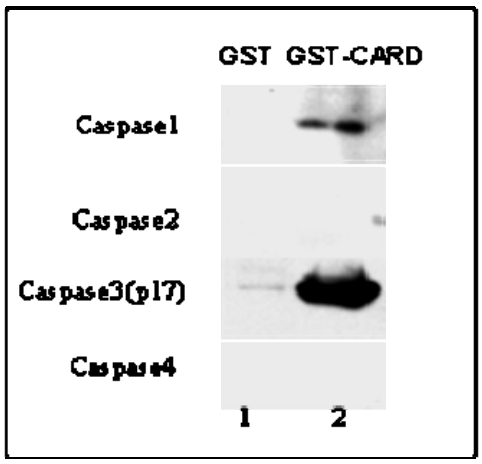


Figure 21: DC-CASPIC interacts with caspase 1 and caspase 3 *in vitro*

The CARD domain at the N-terminus of DC-CASPIC was cloned into a pGEX-KG vector and expressed as GST-tagged CARD domain (GST-CARD). GST (*lane 1*) or GST-CARD (*lane 2*) was used as bait to pull down potential interacting partners from the total DC cell extract, followed by a Western blot analysis using caspase 1, 2, 3 and 4 antibodies.

secondary antibody respectively. Cellular localisation of NOS2 and cytochrome *c* was subsequently detected by confocal immunoflorescence microscopy. However, interestingly, NOS2 only co-localises partially with cytochrome *c* and a major pool of the NOS2 in DC localises to the cytoplasm (Figure 22).

Unexpectedly, we observed that NOS3 co-localises well with cytochrome *c* in the mirochondria under immunoflorescence microscopy (Figure 23A). To extend the confocal immunoflorescence microscopy results with NOS3 to electron microscopy, ultrathin sections of DC2.4 cells were stained with antibodies specific for NOS3 and cytochrome *c*, and labelled with 10nm and 5nm immunogold conjugated secondary antibodies respectively. Their co-localisation was further verified by immunogold electron microscopy. The results indicated that both NOS3 (stars) and cytochrome *c* (arrows) localised in the mitochondria, although NOS3 was also found in the cytosol (Figure 23B).

3.2.12 NOS3 is shown as one of the probable upstream factors regulating DC-CASPIC protein expression

Dawn *et al*. (2002) and Zhao *et al.* (2007) demonstrated that NOS3 is required for the production of NOS2 protein and NO synthesis in postischemic myocardium condition. In their study, the expression of NOS2 protein and mRNA was significantly reduced in heart tissues of NOS3-knockout mice. The study suggests that NOS3 catalyses production of NO which induces NOS2 synthesis via a PKC-dependent signalling pathway (Dawn *et al*., 2002). In conjunction with the study and in view of our observation that LPS induces mRNA and protein expression of NOS3 and DC-CASPIC, it is therefore interesting to examine if the

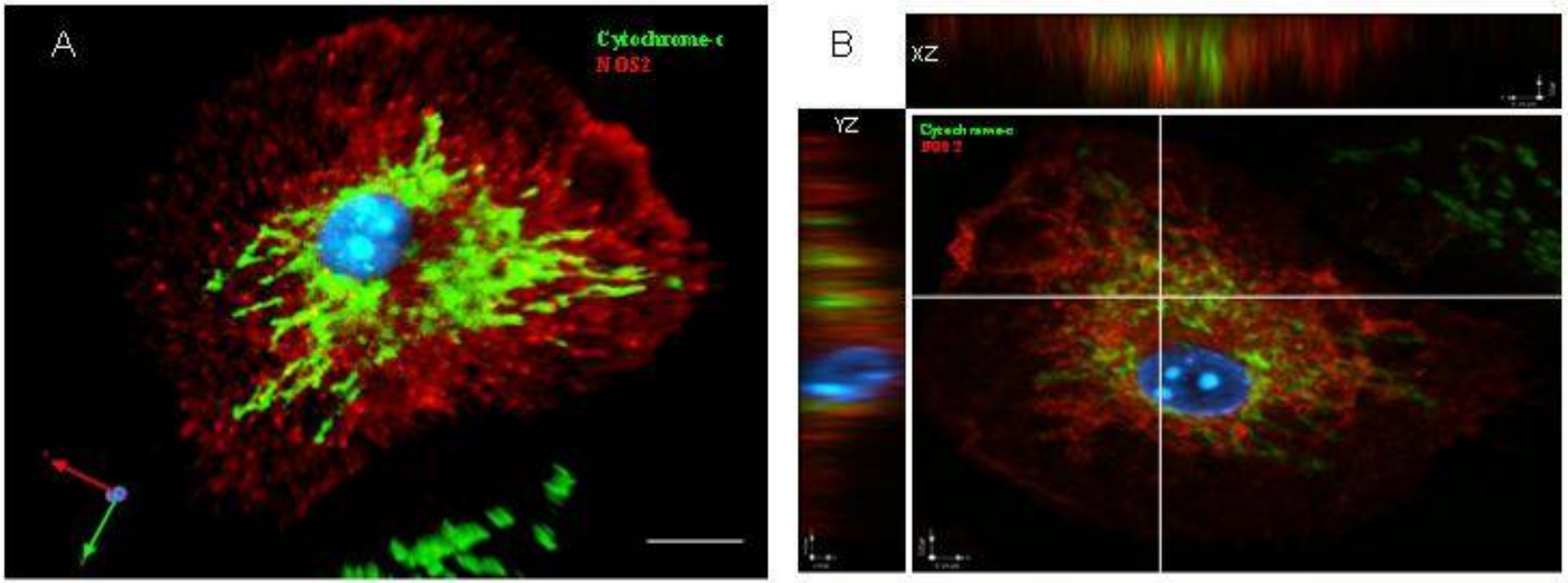


Figure 22: NOS2 does not co-localise in mitochondria

Immature DCs were induced with LPS, then fixed with methanol and stained with antibodies against cytochrome *c* and NOS2 followed by FITC and cy3-conjugated secondary antibodies, respectively. The nucleus was stained by DAPI. NOS2 and cytochrome *c* (Cyto *c*) were subsequently detected by confocal microscopy. (A) Stereo 3D rendering images of cytochrome *c* (green) and NOS2 (red) subcellular localisation in DCs (Scale bar, 10 μm). (B) X-Z/Y-Z projection of collected confocal images showing localisation of NOS2 with cytochrome *c*.

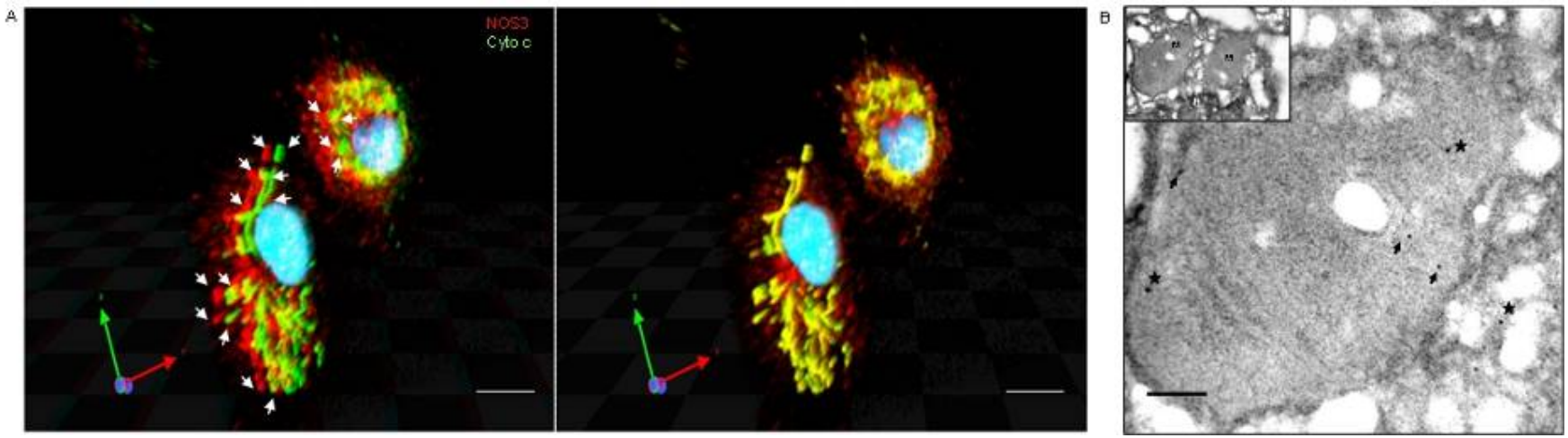


Figure 23: NOS3 localises to mitochondria

(A) Immature DCs were induced with LPS then fixed and stained with antibodies against NOS3 and cytochrome *c* followed by FITC and cy3-conjugated secondary antibodies, respectively. The nucleus was stained by DAPI. NOS3 and cytochrome *c* (Cyto *c*) were subsequently detected by confocal microscopy. Arrows indicate the co-localisation of NOS3 and cytochrome *c*. (Scale bar: 10 μm) (B) Ultrathin section of DC2.4 were fixed and stained with antibodies against NOS3 and cytochrome *c* followed by 5nm and 10nm immunogold-conjugated secondary antibodies, respectively. NOS3 (*) and cytochrome *c* (←) were subsequently detected by electron microscopy (M: mitochondria, N: nuclear, *: NOS3; ←: cytochrome *c* ; Scale bar: 100 nm).

expression of DC-CASPIC is affected in NOS3-deficient DCs upon LPS induction. To test this possibility, DCs from the wild type and $NOS3^{-/-}$ mice were induced with or without LPS. The protein extracts were subjected to Western blot analysis. As shown in Figure 24A, LPS treatment increased the DC-CASPIC protein level in the wild type cells significantly (*lane 1 and 2,* $p < 0.01$). Nevertheless, the induction of DC-CASPIC upon LPS treatment was diminished in NOS3-knock out mice (*lane 3 and 4*, $p > 0.05$). Moreover, knockout of NOS3 reduces the expression of DC-CASPIC by 43% in mature DCs (*lane 2* and *4*, $p < 0.01$), but this effect was less pronounced in immature DCs (*lane 1 and 3*, $p > 0.05$). Thus, NOS3 indeed plays an important role in regulating DC-CASPIC expression during maturation of DCs(Figure 24 A–B).

Next, we examined whether there is a feedback mechanism in the NOS3-mediated regulation of DC-CASPIC by investigating whether DC-CASPIC enhances NOS3 expression during maturation of DCs. Myc-DC-CASPIC was over-expressed in DC2.4 cells and induced with LPS. Myc-DC-CASPIC was then detected with anti-myc antibody in Western blot analysis. As shown in Figure 24 C and D, NOS3 was consistently up-regulated in both the control pDMyc vector and Myc-DC-CASPIC transfected cells upon LPS induction. However, DC-CASPIC over-expression does not seem to enhance NOS3 protein expression in DCs (Figure 24 C, D).

Taken together, our results showed that NOS3 might act upstream of DC-CASPIC, enhancing DC-CASPIC expression during maturation of DCs. In addition, there is a possibility that the regulation of DC-CASPIC by NOS3 could be at the translational level.

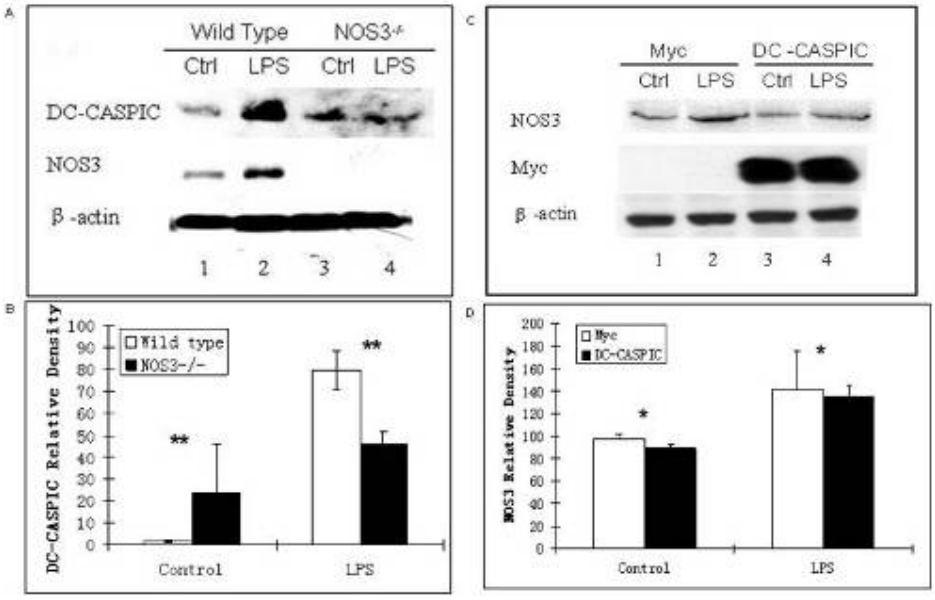


Figure 24: NOS3 identified as one of the probable upstream factors regulating DC-CASPIC protein expression

(A) Wild type and NOS3-knockout DCs were treated or untreated with LPS. Protein extracts were then separated in SDS–PAGE and probed with DC-CASPIC, NOS3 and *β*-actin antibodies in a Western blot. (B) Bars represent the relative density of DC-CASPIC bands in the Western blot analysis (normalised to *β*-actin bands). Data shown were the means ± *s.d.* from three independent cultures. (C) Empty pDMyc or Myc-DC-CASPIC constructs were transfected into DC2.4 cells and the cells were either treated or left untreated with LPS. Protein extracts were subjected to SDS-PAGE and analysed by myc, NOS3 and *β*-actin antibodies in a Western blot. (D) Bars represent the relative density of NOS3 bands in the Western blot analysis (normalised to *β*-actin bands). Data shown were the mean ± *s.d.* from three independent cultures. (**, $p < 0.01$; *, $p > 0.05$)

3.2.13 Over-expression of DC-CASPIC enhances antigen presentation capability of DCs

DC-CASPIC is up-regulated during LPS-induced maturation of DCs. The up-regulation of DC-CASPIC in turn increases NO production through its enhanced interaction with caspases, thus preventing the NOS2 from being degraded by caspases. In our working model, although DC-CASPIC is up-regulated during LPS-induced maturation of DCs and issues in the series of events as described above, it is unclear whether DC-CASPIC initiates maturation of DCs in the absence of LPS.

To address this issue, immature DCs were first transfected with mRNA encoding DC-CASPIC, followed by flow cytometric analysis of several mature DCs surface markers such as MHC class II, CD80 and CD86. As shown in Figure 22, the transfection of DC-CASPIC mRNA into cells increased the cell surface expression of MHC class II (G mean value increased from 32.83 to 53.21) as well as both the essential co-stimulatory molecules CD80 (from 3.95 to 5.33) and CD86 (from 4.32 to 5.66) in the absence of LPS. Thus, DC-CASPIC promotes DCs to display maturation phenotypes (Figure 25).

Besides increasing the cell surface expression of various markers, the functional capability to stimulate T cell proliferation is another critical characteristic of mature DCs. Next, we further examined whether DC-CASPIC-induced mature DCs could stimulate T cell proliferation.

Immature DC2.4 cells were trnsfected with either pDMyc control vector (Myc) or Myc-tagged DC-CASPIC (DC-CASPIC), and then incubated with T

lymphocytes. At day 3, IL-2 produced and secreted by T cells into the culture medium were quantified by ELISA. As shown in Figure 26A, DC-CASPIC enhanced DC-dependent T cell proliferation (IL-2 level) in the absence and presence of LPS. Thus, like LPS- and NO-donor-treated DC, immature DC-over-expressing DC-CASPIC enhances the capability of DCs in inducing T cell proliferation/activation. Figure 26B showed the expression of DC-CASPIC-Myc proteins in DC2.4 transfected cells.

Thus, not only does DC-CASPIC promote maturation phenotypes in DCs, these DCs are also able to functionally stimulate T cell proliferation. Taken together, we have shown that DC-CASPIC initiates maturation of DCs.

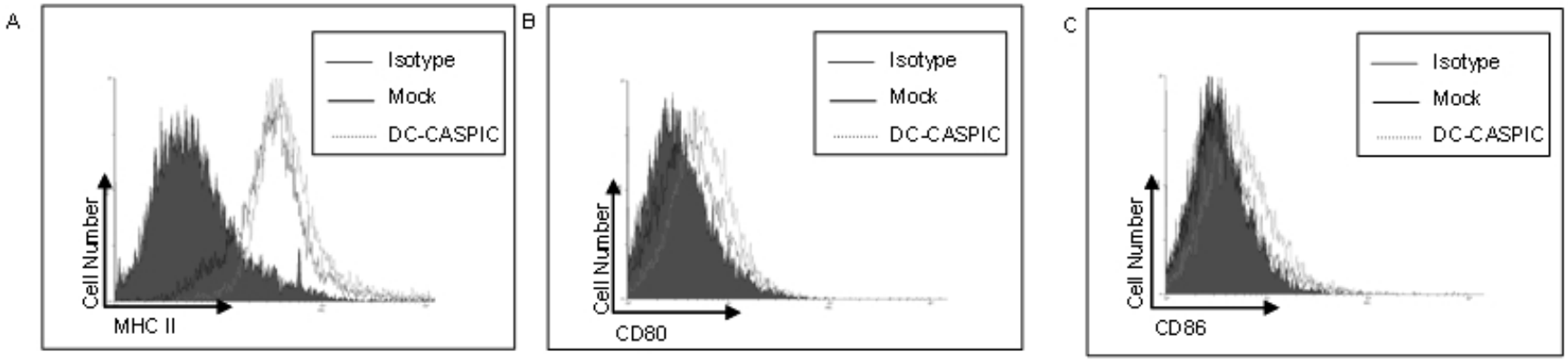


Figure 25: Over-expression of DC-CASPIC enhances DCs surface markers

Immature DCs were transfected with mRNA encoding DC-CASPIC. DCs were stained with (A) MHC class II-FITC antibody (Isotype: mouse IgG_{2a}, Clone: 25-9-17), (B) CD80-FITC antibody (Isotype: mouse IgG_1, Clone: L307.4) , or (C) CD86-FITC antibody (Isotype: mouse IgG_{2a}, Clone: FUN-1) . Stained DCs were analysed by flow-cytometry. Control staining (gray histograms) was performed with the corresponding isotype IgG, Mouse IgG2a for MHC class II, and mouse IgG1 for CD80 and CD86.

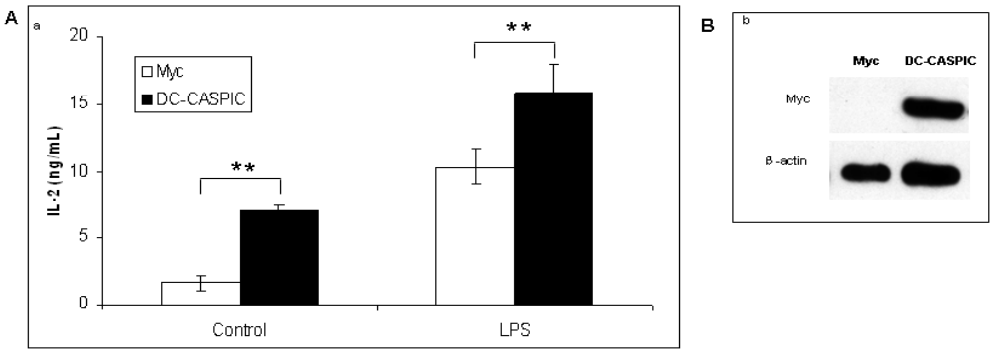


Figure 26: DC-CASPIC enhances DC-dependent T cell proliferation *in vitro*

DC 2.4 cells were either transfected with a pDMyc empty vector or with DC-CASPIC-Myc. These cells were either uninduced or induced with 1μg/mL LPS for 24 hours and subsequently co-cultured with T cells isolated from BALB/c mice in the medium for 72 hours. (A) Quantification of IL-2 in the medium. (B) Cell lysates were subjected to a Western blot analysis using anti-Myc and -β-actin antibodies. Statically significant differences (P-value): **, $p < 0.01$.

3.3 Discussion

3.3.1 Possible type of DCs expressing DC-CASPIC *in vivo*

The expression of DC-CASPIC is under the control of promoter I of CIITA, which is specifically activated in DCs (Muhlethaler-Mottet *et al.*, 1997). There are three categories of DCs, TipDCs, cDCs and pDCs. Our results show that DC-CASPIC may be specifically expressed in TipDCs, but not in cDCs or pDCs.

TipDCs are characterised by their detectable levels of TNF-α, NOS2 and myristoylated alkaline-rich protein kinase C substrate (MAC), their intermediate cell surface expression level of CD11c, a high expression level of CD11b and the absence of CD4 or CD8 expression. The development of TipDCs is dependent on GM-CSF. In the mice that are genetically deficient in the GM-GSF receptor, monocytes which are precursors of DCs, fail to develop into MHC class II positive TipDCs generations, even though they had entered the spleen of the inflamed recipients (Shortman and Naik, 2007). *In vitro,* TipDCs can be cultured from mouse bone marrow-derived or human monocytes that are stimulated with GM-CSF (Randolph *et al.*, 2002; Nikolic *et al.*, 2003). Our results also show that DC-CASPIC expresses in this type of cells.

In addition, the expression of DC-CASPIC in various tissues using a Western blotting analysis revealed that it is mainly expressed in the kidney, liver and small intestine. Accordingly, TipDCs has been shown to express in kidney, liver and intestine of a mouse. In the kidney, although the dichotomy between DCs and macrophages was not clear, most of the $CX3CR1^{+}$ cells were identified as DCs. This type of DCs exhibits high expression level of MHC class II, CD11b

and CD11c in kidney infection that are similar to TipDCs (Kurts, 2006). In the liver, $CD11b^{+}CD11c^{+}$MHC class II^{lo} hepatic DCs are found in mouse (Lau and Thomson, 2003). In addition, mouse hepatic non-parenchymal cells (NPCs) are also able to form mouse bone marrow-derived DCs in the culture medium supplemented with GM-CSF (Randolph *et al.*, 1999). These studies may suggest the existence of TipDCs in the kidney and liver. In the small intestine, according to Randolph's model, migratory monocyte-derived DCs are induced by the inflammatory stimulus and resemble TipDCs, although such kinds of DCs are CD8α- and characteristically have a lower CD11c expression (Lu *et al.*, 1994).

Tissue survey results show that DC-CASPIC is absent in the thymus and spleen, which are rich in cDCs (Ardavin, 2003a); therefore DC-CASPIC does not seem to be expressed in cDCs. DC-CASPIC is also probably not expressed in pDCs, as promoter I which is necessary for the transcription of DC-CAPSIC is inactive in pDCs (LeibundGut-Landmann *et al.*, 2004).

Besides TipDCs, DC-CASPIC has also been found to express in mouse brain, and was detected in a N9 microglia cell line and brain extract.

3.3.2 DC-CASPIC and NOS

An association of NOS-like proteins with mitochondria has previously been demonstrated immunohistochemically (Kobzik *et al.*, 1995; Bates *et al.*, 1996). Further supporting evidence is provided by the detection of nitric oxide synthase activity in mitochondria (Tatoyan and Giulivi, 1998). Consistent with these studies, our Western blot and confocal analysis results using a monoclonal NOS3 antibody (immunogen: amino acid residues 1030–1209) also reveal the

existence of NOS in mitochondria. In accordance with our results, a study showed the docking of NOS3 to the cytoplasmic face of mitochondria as revealed by immunogold electron microscopy (Gao *et al.*, 2004). Further their studies are in consistent with our observations, which is that most NOS3 proteins co-localised with cytochrome *c* in the mitochondria. The co-localisation of NOS3 and cytochrome *c* may also facilitate the interaction of these two molecules. For example, the NOS3 product NO has been reported in previous studies to bind directly and reversibly to cytochrome *c* oxidase. Such binding occurs in competition with oxygen, resulting in the inhibition of cytochrome *c* oxidase activity (Cleeter *et al.*, 1994; Clementi *et al.*, 1998).

The localisation of NOS3 and DC-CASPIC in mitochondria may also provide the possibility of their interactions. Indeed, there is less DC-CASPIC protein in $NOS3^{-/-}$ DCs than in the wild type DCs (as seen in two independent experiments with $p < 0.05$). Moreover, the increase of DC-CASPIC is accompanied with increase in NOS3 in the DCs, Therefore, these results may suggest that the expression of DC-CASPIC in the DCs is at least partially regulated by NOS3.

NOS3 is also responsible for the synthesis of NO. NO has previously been reported involved in the regulation of protease enzymes activities. For instance, NO inhibits caspase activity and causes the increase of syntaxins, adaptins, vti1a and vti1b protein levels in mature DCs (Wong *et al*, 2004). In macrophages, the exposure of cells to *S*-nitrosoglutathione and the excess stimulation of endogenous NO production could result in the inhibition of ubiquitin-proteosome system-associated proteins such as parkin (E3 ligase) and Uch-L1 (de-ubiquitinating

enzyme that recycles ubiquitin) activity (Glockzin *et al.*, 1999). Therefore, it is possible that NOS3 regulates DC-CASPIC expression either by inhibiting caspase or by the ubiquitin proteosome system, which is carried by its product, NO. The screening of the DC-CASPIC protein sequence did not reveal any potential caspase cleavage sites on the DC-CASPIC, but a computer analysis of the DC-CASPIC protein sequence with the program PESTFIND revealed one potential PEST sequence (^{64}KGDLDLTFLSWVCNSLQAPTVE^{86}), which is a ubiquitination target (Rogers *et al.*, 1986; Rechsteiner and Rogers, 1996). Therefore, the degradation of DC-CASPIC in the mitochondria might be regulated by the ubiquitin-proteosome system, which is also found to localise to mitochondria in DCs (Liu *et al.*, 2003). Moreover, ubiquitination is also one of the post-translational mechanisms of DC-CIITA, which derives from the same promoter as DC-CASPIC (Towey and Kelly, 2002; Schnappauf *et al.*, 2003). Thus, it is possible that NOS3 protects DC-CASPIC from ubiquitination in DCs. Further experiments to prove the existence of ubiquitination on DC-CASPIC may be needed to confirm this hypothesis.

NOS2 is another major source of NO in mature DCs. Although NOS2 expression can be regulated primarily at a transcriptional level (Xie *et al.*, 1994; de Vera *et al.*, 1996), translational and post-translational regulations are also important for NOS2 expression and function. Several proteins such as glucocorticoids, caveolin-1 and heat shock protein 90 (hsp90), have been reported to interact with NOS2 and cause NOS2 degradation (Kone *et al.*, 2003). These post-translational regulations on NOS2 probably occur in or near the mitochondria since hsp 90 is reported to localise in mitochondria (Kang *et al.*, 2007), and caveolin-1 is also shown to associate with mitochondria (Mellgren,;Li *et al.*, 2001).

Our findings showing the existence of DC-CASPIC in mitochondria suggest its possible role in regulation of NOS2 activity. Similar to hsp90 and caveolin-1, the results in this study reveal that DC-CASPIC may also regulate NOS2 in or in the vicinity of the mitochondria.

Additionally the results indicate that NOS2 protein expression is indirectly regulated by NOS3, which is able to enhance DC-CASPIC expression. This hypothesis is supported by previous studies, showing that the expression of NOS2 is partially dependent on NOS3 in LPS-induced macrophages (Connelly *et al.*, 2001; Connelly *et al.*, 2005).

3.3.3 DC-CASPIC and caspase family proteins

In this study, the incubation of GST-CASPIC with DC2.4 cell lysate decreases the activities of caspase 1 and caspase 3 but not caspase 8. The protein interaction assay also shows that GST-tagged DC-CASPIC but not GST alone is able to pull down caspase 1 and caspase 3 *in vitro*. One interpretation of these results is that the binding of DC-CASPIC to caspases, especially to caspase 1, inhibits the activities of the latter. The decrease of caspase activities then partially prevents the degradation of NOS2 which contains at least two conserved potential caspase recognition/cleavage sites: ^{847}DETD^{850} for caspase 3 and ^{14}YQSD^{17} for caspase 1 (Luschen *et al.*, 1998). NO in turn acts as a caspase inhibitor to uphold the amount of NOS2 protein in the DCs.

As a CARD domain-containing protein, DC-CASPIC may also interact with other CARD-containing proteins. There are at least three categories of CARD-containing proteins that are involved in immune responses, namely

nucleotide-binding oligomerisation domain proteins (NOD) (NOD1, NOD2, CIITA), Retinoic acid-inducible gene-I (RIG-I), Melanoma differentiation gene 5 (MDA5), Dexh box polypeptide 58 (DHX58) and caspases.

NOD proteins are mainly involved in the LPS induced-DC maturation pathway. For example, NOD1 is a cytoplasmic receptor for LPS. The purified NOD1 protein was originally found to associate with a radiolabelled LPS fraction from *E.coli*, and the binding of the NOD1 to LPS is required for the activation of NF-κB (Inohara *et al.*, 2001). The mutational analysis demonstrated that the CARD domain of NOD1 is necessary and sufficient for the activation of NF-κB, implying that the CARD domain of NOD1 protein functions as an effector domain (Inohara *et al.*, 1999). Moreover, the interaction of NOD1 with RICK is also dependent on the CARD domain, which is critical for the activation of the NF-κB pathway (Chin *et al.*, 2002). However, the possibility of DC-CASPIC interacting with the NOD protein is low. In DC2.4, DC-CASPIC up-regulated the expression of NOS2 at the translational but not at the transcriptional level, as is shown by RT-PCR.

Retinoic acid-inducible gene I (RIG-I) is responsible for the reorganisation of intracellular viral dsRNA (Yoneyama *et al.*, 2004). The importance of the RIG-I pathway in antiviral immunity was confirmed by the generation of RIG-I-deficient mice (Kato *et al.*, 2005), which revealed that RIG-I, but not the TLR system, plays an essential role in the IFN-mediated antiviral response in most cell types, including conventional DCs. RIG-I contains two CARD domains at its C-terminus. The CARD domains of RIG-I interact with the adaptor protein MAVS (mitochondrial antiviral signalling) and activate its downstream NF-κB pathways

(Hiscott *et al.*, 2006). *MAVS* gene also encodes a CARD domain (Seth *et al.*, 2005). A mutational analysis revealed that the CARD domain was essential in signalling to IRF-3 and NF-κB. Expression of the CARD region alone was not sufficient for the induction of IFN-β in response to a viral infection, and a mutant lacking the CARD domain exhibited a dominant negative phenotype for a virus or the dsRNA signalling of the host response (McWhirter *et al.*, 2005). Besides containing a CARD domain, MAVS shares two other similar characteristics with DC-CASPIC. First, MAVS was also found to localise to the mitochondria, and was detected in a detergent-resistant mitochondrial fraction upon viral infection. Its association with mitochondria is necessary for MAVS signalling activity (Yang *et al.*, 2007). Second, MAVS also has a protective role for the cells, because the knockdown of *MAVS* gene expression by siRNA enhances apoptosis in DCs (Seth *et al.*, 2005). The co-immunoprecipitation of DC-CASPIC and MAVS may shed more light on the involvement of DC-CASPIC in the RIG-I-MAVS pathway and in the understanding of the physiological functions of DC-CASPIC.

An intact CARD domain (α-helices 1 to 6) is necessary for CARD-CARD domain interaction (Qin, H., Srinivasula, S. M., 1999). Nevertheless, our data show that the deletion of α-helices 5 and 6 on the C-terminal CARD domain does not affect the proper localisation in the mitochondria and the function of DC-CASPIC in enhancing NO production. These results possibly indicate that DC-CASPIC does not carry out its function through CARD-CARD domain interaction. Therefore, the screening of interacting partners of DC-CASPIC should not be limited only to the CARD domain-containing proteins.

3.3.4 DC-CASPIC and DC-CIITA

It is also imperative to determine if an over-expression of DC-CIITA will result in an increase in the level of NO in the DCs. However, DC-CIITA is likely to bind to caspases and its over-expression is also likely to cause an increase in NO production. The idea that the major part of the DC-CASPIC protein is present only in DC-CIITA but not in type III and type IV CIITA, yet with all three are being able to transactivate the same set of genes such as MHC class II, CD74 and H2-M, suggests that this CARD domain may be distinct and independent from the other functional domains that make up the 3D conformation of type III and type IV CIITA. Thus, the CARD domain will still be able to bind to caspases and regulate their activities in the native form of DC-CIITA. Despite the extensive work done on CIITA to date, the localisation of DC-CIITA has not been investigated and it will be interesting to determine if it localises to the mitochondria. Localisation studies on CIITA thus far have focused only on the type III human CIITA (1130aa) which does not contain the CARD-like domain and which it has been reported to be found both in the nucleus and the cytoplasm (Cressman *et al.*, 2001). The C-terminal two-thirds, which are shared among all CIITA proteins, contain at least two nuclear localisation signals (NL2 and NL3) and are implicated in localisation to the nucleus. The presence of these signals in the full-length DC-CIITA might thus override those present in the CARD-like domain, affecting its localisation to the mitochondria. However, it is interesting to note that the GTP-binding domain found in CIITA has been reported to regulate its nuclear export (Raval *et al.*, 2003) and that the cellular localisation of CIITA is tightly controlled. This has been suggested to act as a regulator for the maintenance of steady-state levels of MHC class II in APCs. This also provides a

reservoir of CIITA to affect a rapid increase in *MHC* gene expression in response to changes in the external milieu. If CIITA indeed plays multiple functions besides being a transactivator, the reason for this tightly controlled cellular localisation of CIITA becomes apparent. The interaction of DC-CIITA and caspases (if proven) should occur in the cytoplasm and therefore the cellular distribution of CIITA should be a dynamic one that is determined by its various translocation signals.

3.3.5 The functions of DC-CASPIC

MHC class II and related genes are the most important target genes of CIITA. In addition to the genes encoding classic MHC class II molecules, CIITA activates the expression of several genes, encoding accessory proteins required for an MHC class II-restricted antigen presentation such as CD74 and H2-M (Chin *et al.*, 1997; Nickerson *et al.*, 2001; Masternak and Reith, 2002). This clearly remains as the primary function of CIITA. However, a series of recent reports have suggested that CIITA may also be implicated in other functions within and outside the immune system. In one report, the *collagen α2 (I)* gene has been reported to be repressed by CIITA (Xu *et al.*, 2008). Furthermore, in another report, the semaphorin receptor plexin-A1 was observed to express abundantly in mature DCs, and CIITA plays essential roles in modulating its expression (Wong *et al.*, 2003). This observation is particularly interesting as plexin-A1 expression was found to enhance the ability of DCs to promote T cell stimulation. Here, we show that DC-CASPIC, a novel splice-isoform of the DC-CIITA that mainly encodes the entire CARD-like domain of DC-CIITA, can modulate NOS2 protein expression, NO synthesis and antigen presentation in DCs. In our model, DC-CASPIC protein expression is low in immature DCs. However, upon LPS

`induction (mature DCs), the protein expression of DC-CASPIC is induced by NOS3, and DC-CASPIC interacts with caspases. In addition the interaction of DC-CASPIC with caspases inhibits caspase activity and indirectly up-regulates the protein expression of NOS2 and possibly other mitochondrial nitric oxide synthase, and thus increases intracellular NO synthesis. Through a positive feedback system, NO then further inhibits caspases and enhances the production of more NOS2 protein in the DCs. The inhibition of caspases by NO and other endosomal proteins is essential for regulating the MHC class II-restricted antigen presentation pathway in DCs and T cell proliferation (Wong *et al.*, 2004; Santambrogio *et al.*, 2005). Thus, this study strongly suggests that DC-CASPIC plays important roles in regulating the antigen-presenting capability of DCs during maturation (Figure 27).

3.3.6 Limitations and future direction

In this part of the study, a novel protein, DC-CASPIC, which is specifically expressed in DCs, has been investigated. DC-CASPIC, which is specifically expressed in the mitochondria of DCs, is mainly composed of a whole CARD-like domain plus an extra stretch of 28 amino acids with no homology to any of the CIITA isoforms. During LPS-induced maturation, the DC-CASPIC protein is induced by the up-regulation of NOS3. The up-regulated DC-CASPIC may be able to slow down the degradation of NOS2 by inhibiting activities of caspases, especially caspase 1 and caspase 3, through CARD-CARD domain interaction, thus resulting in an increase in the level of NO. The increase of NO level then promotes the maturation of DCs, which is observed in DC-CASPIC over-expressing cells. However, there are still several limitations in this project.

First, both DC-CASPIC and DC-CIITA are transcripted from same promoter and share same precursor mRNA. Therefore, it is rather technically challenging to further confirm the roles of DC-CASPIC in antigen presentation and T cell activation by specifically knockdown DC-CASPIC using siRNA transfection without affecting the expression of DC-CIITA. Second, it would be much more informative to have DC-CIITA full-length protein as control in all of tissue survery, cellular location and protein function studies. However, unavailability of DC-CIITA specific antibodies hinders these experiments. Third, our results revealed that DC-CASPIC localised in mitochondria by using overe-expression of myc-tagged DC-CASPIC. And this result is further confirmed by cell fractionation experiment which showed that DC-CASPIC was enriched in mitochondrial fraction in DCs. Unfortunately, the DC-CASPIC antibody we raised is not able to stain the endogenous DC-CASPIC although this antibody successfully detects endogenous DC-CASPIC in Western blot. This may due to the epitode is enclosed in whole cells but open after cell is lyesed. However, it will be great interesting to directly show the mitochondrial localisation in other types of DCs such as *bonafide* DCs. In addition, the mutagenesis study showed that H3 and H4 helices are necessary for DC-CASPIC mitochondrial localisation and for its enhancement on NO production. Nevertheless, the possibility cannot be excluded that deletion of helices after helices from an integrated domain can generate artificial binding sites or destroy the overall integrity of the domain. It might not tell which region is involved in binding. A point mutagenesis study at residue level would be much more informative. Fourth, although the initial localisation study has led to the discovery of several interacting partners of DC-CASPIC, such as NOS3, NOS3, caspase 1 and caspase 3, there may still be other

potential interacting partners in the cells. A pull-down experiment followed by a protein sequence analysis will help to screen out the potential candidates. Moreover, even though the pull-down assay and immunoprecipitation show the binding of DC-CASPIC to the caspases, the detailed mechanism of this binding is still unclear. Future studies on the truncation mutation and crystal structure of DC-CASPIC may provide insights into this question.

In conclusion, this study on DC-CASPIC demonstrates that DC-CASPIC is one of the key molecules that regulate NO synthesis and antigen presentation during maturation of DCs. A novel regulatory pathway has been demonstrated on DC-CASPIC in antigen presentation by DCs. Further identification of DC-CASPIC functions with its role in maturation of DCs would provide a new biomarker with potential therapeutic intervention.

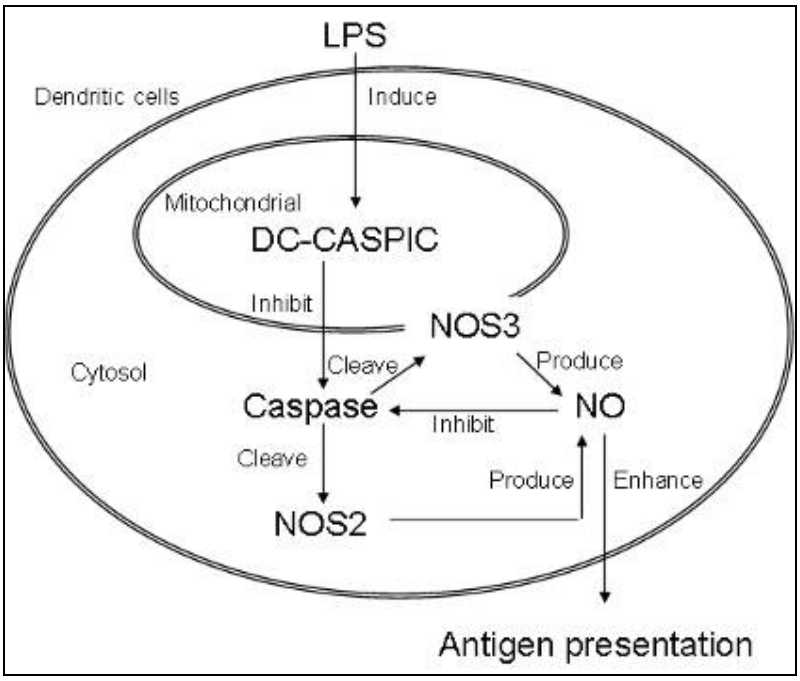


Figure 27: Interactions of DC-CASPIC with NOS2 increase NO production and antigen presentation capability of DCs

CHAPTER 4

NOS2 INTERACTS WITH CD74 AND INHIBITS ITS CLEAVAGE BY CASPASE DURING DENDRITIC CELL DEVELOPMENT

4.1 MATERIALS AND METHODS

4.1.1 Mice

C57/BL 6J wild type, $NOS3^{-/-}$ and $NOS2^{-/-}$ mice ($I\text{-}A^b$, $I\text{-}E^b$) were purchased from Jackson Laboratory (Bar Harbor, USA). BALB/c wild type mice ($I\text{-}A^d$, $I\text{-}E^d$) were purchased from centre for animal resources Singapore. All protocols on mice are conducted under the Institutional Animal Care and Use Committee (IACUC), National University of Singapore.

4.1.2 Cell lines and cell culture medium

Mouse dendritic cell line, DC2.4, was kindly provided by Dr. Kenneth Rock (University of Massachusetts Medical Centre, Worcester, USA). Mouse dendritic cell line JAWSII and microglia cell line N9, was obtained from the American Type Culture Collection (ATCC) (Manassas, USA). Dulbecco's Minimal Eagles medium (DMEM medium), Opti-MEM, Fetal bovine serum (FBS) and MEM non-essential amino acids were purchased from Invitrogen (Carlsbad, USA). Effectene transfection reagents used for transfection were purchased from Invitrogen (Carlsbad, USA). Tissue culture flasks, plates and other disposables were either from Nunc (Rochester, USA).

4.1.3 Antibodies and other reagents

N^G-Monomethyl-$_L$-arginine ($_L$-NMMA), spermine NONOate, lipopolysaccharide, *E. coli* (LPS), caspase inhibitor I, caspase 1 inhibitor, caspase I inhibitor VI, caspase-5 inhibitor 1, caspase 3 inhibitor III and Carboxy fluoroscein succinimidyl ester (CFSE) were purchased from Calbiochem

(Darmstadt, Germany). Granulocyte/Macrophage colony stimulating factors (GM-CSF) and macrophage colony stimulating factors (M-CSF) were purchased from R&D Systems (Minneapolis, USA). Greiess reagent was purchased from Sigma-Aldrich (St. Louis, USA). The chromatic caspase activity kit was purchased from Calbiochem (Darmstadt, Germany). Ficoll-paque plus was purchased from GE Healthcare (Bjőrkgatan, Sweden). Other chemical reagents which are not mentioned here are all from Sigma-Aldrich (St. Louis, USA).

Protein A/G coupled sepharose beads were purchesed from Amersham Biosciences (Buckinghamshire, UK). Reduced glutathione and glutathione agarose beads were purchased from Sigma-Aldrich (St. Louis, USA).

QIAquick gel extraction kit, Miniprep DNA Purification Kit and Maxi DNA Purification Kit were purchased from Qiagen (Hilden, Germany).

Restriction enzymes, T4 DNA ligase, *Taq* DNA polymerase, *Pfu* DNA polymerase, T7 or SP6 polymerase, M-MLV reverse transcriptase, and calf intestine alkaline phosphatase (CIP) were purchased from Promega (Madison USA). A-Plus polymerase was purhcased from Epicentre Biotechnoloies (Madison, USA). Caspase 1 and caspase 4 enzymes were purchased from Calbiochem (Darmstadt, Germany). 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) was purchased from Vector Laboratories (Burlingame, USA).

The primary antibodies used in this study are listed in table 4. The various goat anti-mouse/anti-rabbit immunoglobulin conjugated to either fluorescein isothiocyanate (FITC), cyanine dyes 3 (Cy3) or rhodamine were purchased from

Jackson Immunoresearch Laboratories (West Grove, USA). The corresponding IgG for isotype control were purchased from BD Biosciences (San Jose, USA). The secondary antibodies, *i.e.* goat anti-mouse / anti-rabbit antibodies (HRP-conjugated) and nitrocellulose membrane were purchased from Amersham Biosciences (Buckinghamshire, UK). West Pico Supersignal substrate/enhancer was purchased from Perice Chemical (Rockford, USA).

Table 4: Antibodies used in CD74 study

Antibody	Isotype	Clone name	Flourochrome	Company
CD74	Rat IgG_{2b}	IN-1	--	BD Biosciences, San Jose, USA
C-myc	Mouse IgG_1	9E10	--	EMD Biosciences, Inc. Calbiochem, USA
NOS2	Mouse IgG_1	2	--	BD Biosciences, San Jose, USA
NOS2	Rabbit IgG	Polyclonal	--	BD Biosciences, San Jose, USA
NOS3	Mouse IgG_1	3	--	BD Biosciences, San Jose, USA
I-A/I-E	Mouse IgG_{2b}	M5/114.15.2	--	BD Biosciences, San Jose, USA
α-adaptin	Mouse IgG_1	8	--	BD Biosciences, San Jose, USA
γ-adaptin	Mouse IgG_1	8	--	BD Biosciences, San Jose, USA
Vti1a	Mouse IgG_1	45	--	BD Biosciences, San Jose, USA
Vti1b	Mouse IgG_1	7	--	BD Biosciences, San Jose, USA
β-actin	Mouse IgG_1	AC15	--	Sigma-Aldrich, Inc. USA
c-myc	Rabbit IgG	Polyclonal	--	Santa Cruz Biotechnology Inc., Cruz, USA
Caspase 1	Rabbit IgG	Polyclonal	--	Santa Cruz Biotechnology Inc., Cruz, USA
Caspase 3	Rabbit IgG	Polyclonal	--	Santa Cruz Biotechnology Inc., Cruz, USA
Caspase 11	Goat IgG	Polyclonal	--	BD Biosciences, San Jose, USA
Syntaxin 8	Mouse IgG_{2a}	48	--	BD Biosciences, San Jose, USA
CD11c	Hamster IgG_1	HL3	FITC	BD Biosciences, San Jose, USA
CD80	Mouse IgG_1	L307.4	FITC	BD Biosciences, San Jose, USA
CD86	Mouse IgG_1	FUN-1	FITC	BD Biosciences, San Jose, USA
$I\text{-}A^b$	Mouse IgG_{2a}	25-9-17	FITC	BD Biosciences, San Jose, USA

4.1.4 Cell culture

4.1.4.1 Culture of DCs

C57/BL 6J wild type and $NOS2^{-/-}$ mice ($I\text{-}A^b$, $I\text{-}E^b$) were sacrificed by CO_2 and sterilised by soaking into 70% ethanol for one minute. After peeling away the fur by forceps, the hand legs were cut out and put into plain DMEM and the muscles were scraped off by forceps and blades. Before filtering with 70 μM mesh nylon screen, the hematopoietic stem cells were flushed out from the resulting long bones with 21 G needle and 1 mL syringe into DMEM supplemented with 5% FBS containing 20 ng/mL of GM-CSF. Then cell number and viability were examined by hemocytometer and trypan blue stain before seeding into cell culture dishes. On day 2, 4 and 5, half of volume of old DMEM medium was replaced with same volume of fresh complete DMEM medium with 15 ng/mL of GM-CSF. The immature DCs were harvest in day 7 by flushing out with blue tips or by 5 μM EDTA. To induce cell maturation, the day five cells were further subcultured in DMEM supplemented with 1 μg/mL LPS (Sigma-Aldrich, St. Louis, USA) for 40–48 hours.

4.1.4.2 Culture of primary macrophages

Bone marrow-derived macrophages were cultured with the same protocol as described in section 4.1.4.1. Instead of GM-CSF, complete DMEM is supplemented with 20 ng/mL M-CSF.

4.1.4.3 Thymocyte isolation

BALB/c mice were sacrificed by CO_2 and sterilised by soaking into 70% ethanol for one minute. Freshly removed thymus was placed in 60 × 15 Petri dishes containing 3 mL complete DMEM. Using a circular motion, the thymus was pressed against the bottom of the Petri dish with the plunger of 5 mL syringe until mostly fibrous tissue remained. Clumps in the suspension were further dispersed by drawing up and expelling the suspension several times through a 5 mL syringe equipped with a 19 G needle. Suspension was expelled into a centrifuge tube through a 200 μM mesh nylon screen. After washing one time with complete DMEM, the cells were resuspended into 16 mL complete DMEM. Six milliliter Ficoll paque phase was layered under the cell suspension carefully followed by centrifugation for centrifuge 15 minutes at 800 *g* at room temperature. No brake was used in centrifugation. Thymocyte cells floating on top of the high-density solution were isolated by moving pipette tip over the surface of high-density layer and by drawing cells up in a 5 mL pipette. Thymocytes were transferred into another tube and washed twice with complete DMEM. Then cell number and viability were examined by hemocytometer and trypan blue stain before seeding into cell culture dishes. Cells were seeded into 24-well plate with a cell density of 3×10^6 cells/well and were maintained in the DMEM medium supplemented with 50 ng/mL of IL-2.

4.1.4.4 Culture of cell lines

DC2.4 and N9 cells were maintained in DMEM supplemented with 10% FBS, antibiotics and L-Glutamine (Invitrogen, Carlsbad, USA). JAWS II cells were maintained in DMEM supplemented with 10% FBS and 2ng/mL IL-2. The

mammalian expression vectors used were either pCDA3.1 or pDMyc (modified pCINeo vector with two copies of Myc tag at the 5' end). Cells were plated into plates or dishes and grown for 24 hours to a confluency of 50–60%. Lipofectamine 2000 (Invitrogen, Carlsbad, USA) and Effectene (Invitrogen, Carlsbad, USA) were used as transfection reagents for DC2.4 cells according to the manufacturers' instructions. Cells were harvested for microscopic examination 20 hours after transfection.

4.1.5 Mix lymphocyte reaction assay

Twenty thousand DCs were co-cultured with 2×10^6 CFSE-labelled T cells. For CFSE labelling, purified thymocytes were resuspended with 0.1% (w/v) BSA in PBS at a density of 5×10^6 cells/mL and were labelled for ten minutes at 37 °C with 0.3 μM CFSE. CFSE-labelled cells were 'quenched' with PBS containing 5% (v/v) FBS and were washed twice with PBS containing 5% (w/v) BSA. T cell division was assessed by FACS analysis after 48 or 72 hours of co-culture.

4.1.6 Molecular cloning

The DNA fragment to be cloned into a specific vector was either amplified by polymerase chain reaction (PCR) or restriction digested from another plasmid DNA. The sequences of the primers used for PCR normally included the sequence recognised by specific restriction enzymes selected for the cloning. Ten microgram of vectors were typically digested overnight at 37 °C by one unit of restriction enzymes and dephosphorylated by one unit of calf intestinal phosphatase for 45 minutes at 37 °C. One unit of ligation was performed using T4

DNA ligase, with the ligation mixture incubated overnight at 4 °C. Transformation was then carried out by incubating the ligation mixture with *E. coli* DH5α competent cells on ice for 30 minutes. Cells were then heat-shocked for one minute in a 42 °C water bath. Lysogeny broth (LB) was then added to the cells and the cells were incubated for 45 minutes at 37 °C for recovery. The cells were then plated out onto LB plates supplemented with 100 mg/mL ampicillin and incubated at 37 ^{o}C overnight. Recombinant clones were verified by automated sequencing.

4.1.7 Reverse transcription

Total RNA was extracted from cells using Trizol reagent (Invitrogen, USA) according to manufacturer's instructions. Total RNA was then recovered by precipitation with isopropyl alcohol. Synthesis of cDNA from mRNA transcripts was performed using the following: 3 μg RNA, 1.5 μg oligo (dT) in a reaction volume of 25 μL. Samples were heated at 70°C for 5 minutes, placed on ice immediately and then treated with 0.5 μL RNase inhibitor, 5 μL MLV-RT buffer, 10 mM dNTP, followed by incubation at 42 °C for two minutes. After a pulse spin, 2 unit of MLV reverse transcriptase was then added to the samples and were incubated at 42 °C for 90 minutes. After the incubation, samples were heated to 70 °C for five minutes and then stored at -20 °C.

4.1.8 PCR-based site-directed mutagenesis

Wild type CD74 recombinant plasmids were used as templates for mutagenesis. Template-specific mutagenic primers were designed for the PCR-based deletion mutagenesis, as shown in Table 5. Briefly, a 50 μL reaction system

containing 5–50 ng wild-type plasmid, 0.25 μM of sense and antisense mutagenic primers respectively, 0.2 μM dNTPs and 2.5 unit of *Pfu* DNA polymerase was subjected to 16 thermal cycles (95 °C for one minute, 55 °C for one minute, and 72 °C for six minutes; final extension at 72 °C for 20 minutes). Fifteen microliter of the PCR products were then digested with one unit of corresponding restriction enzymes and ligated unto respective vectors followed by transformation into *E.coli* DH5α competent cells by heat shock. The mutant sequences were then verified by automated DNA sequencing.

Table 5: Primers used in CD74 study

Constructs	Primer Sequences
Myc-CD74	Sense 5'GGAATTCCATGGATGACCAACGCGACCTCATCTCTAACCAT3' Antisense 5' CTCTAGAGCTCACAGGTGACTTGACCCAG 3'
Myc-CD74-A(D)	Sense 5'GGAATTCCATGGATGACCAACGCGCCCTCATCTCTAACCAT3' Antisense 5' CTCTAGAGCTCACAGGTGACTTGACCCAG 3'

4.1.9 *In vitro* transcription of capped mRNA

The constructs were linearised by digestion with an appropriate restriction endonuclease followed by purification with QIAquick gel extraction kit (Qiagen). The *in vitro* transcription was performed with T7 polymerase according to the manufacturer's instruction (Promega, Madison USA). Briefly, a 50 μL reaction system containing 5 μg linear DNA templates, 25 μM of rNTPs, 0.2 μM dNTPs, 4 μM m^7G Cap analog and 5 U of T7 polymerase was incubated at 37 ^oC for three hours. After that, 8 U of A-plus poly (A) polymerase and 1mM ATP were added to the mixture and further incubated for 30 minutes to generate a poly (A) tail. After performing the *in vitro* transcription reaction, the mRNA was purified by

phenol extraction followed by ethanol precipitation. The RNA concentration was determined by absorbance at 260 nm and visualised by denaturing gel electrophoresis.

4.1.10 mRNA electroporation

DCs were harvested at day six with 2 mM EDTA, washed twice with serum-free Opti-MEM and resuspended to a final concentration of 4×10^7 cells/mL in Opti-MEM medium. Subsequently, 100 μL cell suspension was mixed with 20μg mRNA and electroporated in a 0.2 cm-cuvette (voltage: 300 V, capacitance: 150 μF resistance: 100 Ω) using the GenePulser II apparatus (Bio-Rad). After electroporation, the cells were transferred to fresh culture medium containing GM-CSF or induced with 1 μg/mL LPS. The cells were cultured for 24 hours to allow full maturation.

4.1.11 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were cast with the Bio-Rad miniprotein III gel casting system. The resolving gel contained different percentages of acrylamide/bisacylamide mixture (30% with a ratio of 29:1) in 0.375 M Tris-Cl, pH 8.8 and 0.1% (w/v) SDS. The stacking gel contained 4% (v/v) acrylamide/bisacylamide in 0.125 M Tris-Cl, pH 6.8 and 0.1% (w/v) SDS. Polymerisation was induced by the addition of ammonium persulfate (APS) and N,N,N,N,-Tetramethyl-Ethylenediamine (TEMED). Protein samples were dissolved in SDS sample buffer (50 mM Tris-Cl, pH 6.8, 2% (w/v) SDS, 100 mM dithiothreitol (DTT), 10% (v/v) glycerol and 0.1% (v/v) bromophenol blue) and

loaded onto the gel. Gel electrophoresis was typically carried out in Tris-glycine buffer (0.3% (w/v) Tris-base, 1.4% w/v Glycine and 0.1% (w/v) SDS) at a constant voltage of 100 V.

4.1.12 Western blot analysis

The proteins to be identified were subjected to SDS-PAGE separation and the separated proteins were then transferred onto nitrocellulose membrane. The transfer was carried out in transfer buffer (0.3% (w/v) Tris-base and 1.4% (w/v) glycine) with a Bio-Rad wet transfer apparatus (Bio-Rad Kaboratories, Miami USA) at constant voltage of 100 V for one hour. The membrane was then blocked in 5% (w/v) skim milk followed by incubations with specific primary antibodies and appropriate secondary antibodies conjugated to horse radish peroxidase (HRP) diluted in 5% (w/v) skim milk. Membranes were washed three times with PBS + 0.05% (v/v) Tween 20 after each antibody incubation steps. Immunoreactive signals were visualised using a chemiluminescent substrate (West Pico supersignal Kit, Pierce, Rockford USA) and X-ray films, which were developed using a Kodak X-ray film processor (Rochester, USA).

4.1.13 Immunoprecipitation

Proteins were extracted from cells and incubated on ice for one hour in cold lysis buffer (20 mM Tris [pH 8.0], 10 mM EDTA, 100 mM NaCl, 1 mM DTT, 1% (v/v) Brij 98, a polyoxyethylene ether followed by centrifugation at 13,000 rpm for 15 minutes at 4 ^{o}C. Protein exacts (1 mg) were incubated overnight with 5 μg of the corresponding antibodies bound to protein G Sepharose beads (Pharmacia) in lysis buffer plus 1% (w/v) BSA and 10% (v/v) FBS at 4 ^{o}C.

Beads were then washed three times in Buffer A (20 mM HEPES, [pH 7.2], 100 mM KCl, 1 mM DTT, 10 mM EDTA, 0.2 mM ATP, 0.5% (v/v) Brij98) and three times in Buffer B (identical to Buffer A except without Brij98) before being resuspended in SDS sample buffer. Immunoprecipitated proteins and 5% of total the supernatant were separated on SDS-PAGE and analysed by Western blot.

4.1.14 Paraformaldehyde fixation and immunofluorescence staining

Cells grown on cover glasses were fixed in 4% (w/v) paraformaldehyde for 30 minutes at room temperature. The cover glasses in the 6-well or 24-well plates were then washed (with rocking for 2–3 minutes) once with PBS, twice with PBS supplemented with 100 mM NH_4Cl and once with PBS again. The cells were then permeabilised with 0.1% (w/v) saponin (Sigma, St. Louis, USA) for ten minutes at room temperature. The coverglasses were then incubated with primary antibody followed by secondary antibody (conjugated to fluorophore FITC or Texas-Red) diluted in staining buffer (5% (w/v) BSA, 2 mM EDTA, 10% (w/v) goat serum in PBS). Non-specific binding was removed by washing (with rocking for 2–3 minutes) three times with PBS supplemented with 1% (w/v) saponin. The coverglasses were finally mounted onto glass slides with one drop of Vectashield mounting medium. The edges of the coverglass were sealed with nail polish and the slides were then ready for viewing under a fluorescence microscope. The confocal images were taken using either Olympus Fluoview 500 microscope with Fluoview version 5.0 software (Tokyo, Japan). The conventional fluorescent images were taken using Olympus BX-60 digital microscope with ImagePro Plus software (Tokyo, Japan).

4.1.15 Flow cytometry analysis

Cells were harvested with PBS supplemented with 2 mM EDTA, then washed once with staining buffer (5% (w/v) BSA, 2 mM EDTA, 2 mM NaN_3 in PBS) followed by incubation for 30 minutes on ice with the FITC-conjugated antibody. For intracellular staining, cells were fixed and permeabilised with pre-chilled methanol for two minutes, then incubated with primary antibody followed by PE conjugated anti-rabbit IgG. After washing with staining buffer, cells were fixed with 1% (w/v) paraformaldehyde and the immunophenotypic analysis was performed on a FASCAN flow cytometry (DAKO, Glostrup, Denmark).

4.1.16 Quantification of NO

NO was assayed by measuring the concentration of stable end product NO_2^-. NO_2^- production was determined by Griess reaction. Aliquots of culture supernatant (100 μL) were incubated with 100 μL of Griess reagent (Sigma, St. Louis, USA)at room temperature for ten minutes. The absorbance at 550 nm was then measured in an automated plate reader. NO concentration was determined with reference to a $NaNO_2$ standard curve.

4.1.17 Caspase cleavage assay

Cell were lysed with reaction buffer (100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% (v/v) glycerol and 0.1% (v/v) CHAPS pH 7.4) for five minutes at 4 ^oC. CD74 protein was immunoprecipitated by protein G beads, which were pre-bound with CD74 antibody. After washing twice with reaction buffer, the immunoprecipitated proteins were resuspended in 30 μL reaction

buffer and 10 units of caspase enzyme was added. After three hours of incubation, the mixture was resuspended in SDS sample buffer followed by analysis using SDS-PAGE and Western blot.

4.1.18 Caspase activity assay

Ten million cells were lysed with 1mL lysis buffer (50 mM HEPES, 5 mM DTT, 0.1 mM EDTA, 0.1% (v/v) CHAPS, pH 7.4) for four minutes at 4 ^{o}C. Twenty microgram of protein was incubated at 37 °C in a buffer containing 25 mM HEPES (pH 7.5), 10% (w/v) sucrose, 0.1% (v/v) CHAPS and 10 mM DTT, with the respective colourimetric substrates [caspase 1 substrate VI (Z-YVAD-*p*NA), caspase 2 substrate I (Z-VDVAD-*p*NA), caspase 3 substrate IV (Ac-DEVD- *p*NA), caspase-4 substrate II (Ac-LEVD-*p*NA)] (all from Calbiochem, Darmstadt, Germany), in a 96-well, flat-bottom microtiterplate. The cleavage of the substrates was quantified by spectrophotometric detection of free *p*NA (λ= 400 nm) after cleavage from the caspase peptide substrates after two hours.

4.1.19 Statistics

Two tail-student's *t*-test was used for statistical analyses.

4.2 Results

The studies in Chapter 3 have shown the involvement of DC-CASPIC in enhancing antigen presentation by increasing NOS2 activity and inhibiting caspase activity in DCs. However, some questions in this regulatory mechanism remain open. For example, what is the function of NO during maturation of DCs; and how does the increase in NO production enhance antigen presentation? An iinvestigation into these questions would help to elucidate the function of DC-CASPIC and to understand the antigen presentation regulation in DCs.

4.2.1 Nitric oxide initiation of maturation of DCs

4.2.1.1 The increase of NO in DCs after LPS induction.

An increase in NO production is one of the characteristics in maturation of DCs. There are at least three isoforms of NOS genes in DCs, namely NOS1, NOS2 and NOS3. Both NOS2 and NOS3 have been reported to express in DCs (Weis *et al.*, 2002; Serbina *et al.*, 2008). To identify the gene responsible for the production of NO in maturation of DCs, DCs derived from either NOS2 or NOS3-knockout mice were subjected to LPS-induced maturation and the NO production was analysed by Griess assay. The results showed that the increase in NO production in the *NOS2* gene knockout DCs was fully abolished; whereas in the *NOS3* gene knockout mice, NO production was also significantly subdued ($p < 0.01$) (Figure 28A). The NO donor, NONOate, was used as positive control for NO production. Therefore, the *NOS2* gene was the major gene for the production of NO, although *NOS3* also contributed to NO production. Western blot and RT-PCR assays also indicated that NOS2 and NOS3 were induced during maturation

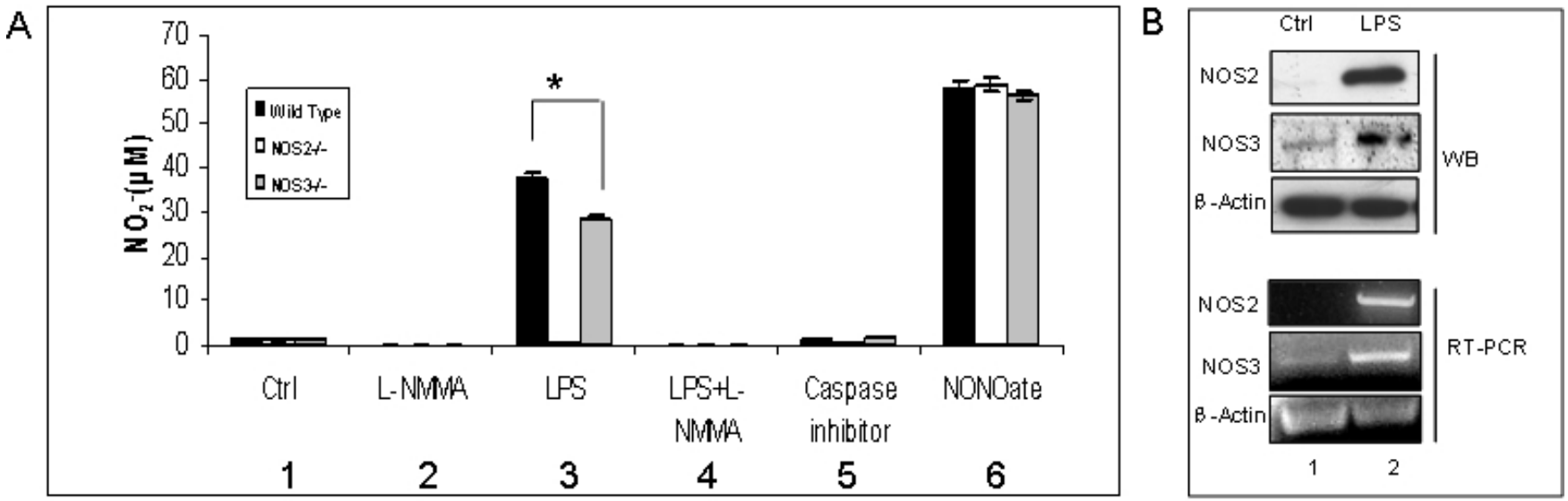


Figure 28: NOS2 produces NO during the maturation of DCs

(A) Immature DCs derived from the wild type, $NOS2^{-/-}$ and $NOS3^{-/-}$ mice were either untreated (group 1) or treated with $_L$-NMMA (group 2), LPS (group 3), $_L$-NMMA/LPS (group 4), or NONOate (group 5). NO produced by untreated and treated wild-type (black bar), $NOS2^{-/-}$ (white bar) and $NOS3^{-/-}$ (grey bar) DCs was detected in a Griess assay. Data shown are the mean ± *s.d.* from three independent cultures. (B) Immature DCs were either untreated (Ctrl) or treated with LPS, and the change in NOS2, NOS3 and *β*-actin expression were detected by a Western blot analysis (upper panel) and RT-PCR (Lower panel) analysis (*, p <0.01).

at both the mRNA and protein levels (Figure 28B). Taken together, our results suggest that both NOS2 and NOS3 contribute to NO production in LPS-induced DCs. Nevertheless, NOS2 was the major NOS that catalysed NO synthesis.

4.2.1.2 NO increases surface expression of MHC class II, CD80 and CD86

During maturation of DCs, NO was produced in large amounts by NOS2, which was also up-regulated during maturation of DCs (Figure 28A). Despite the fact that NOS2 is the major NOS that catalyses the production of NO during LPS-induced maturation of DCs, it is still unclear whether NO alone could contribute directly to the initiation of maturation of DCs and the induction of T cell proliferation in the absence of LPS. To address this issue, immature DCs were incubated with the NO donor (NONOate) alone and stained for cell surface MHC class II, CD80 and CD86 by flow cytometry. Interestingly, we observed that NONOate alone was able to up-regulate cell surface expression of MHC class II as well as both the essential co-stimulatory molecules CD80 and CD86 in the absence of LPS. Furthermore, an increase in surface expression of MHC class II, CD80 and CD86 was also observed in DCs over-expressing NOS2 and NOS3. In this experiment, LPS-treated DCs were used as a control (Figure 29). Thus, NO was able to increase the surface expression of MHC class II, CD80 and CD86.

4.2.1.3 NO up-regulates endosomal proteins in DCs

Since NO can induce higher cell surface expression of MHC class II, CD80 and CD86 on surface of DCs, it would be interesting to determine whether NO could also regulate expression of protein molecules essential for the stability and intracellular trafficking of MHC class II complex in DCs. Previous studies

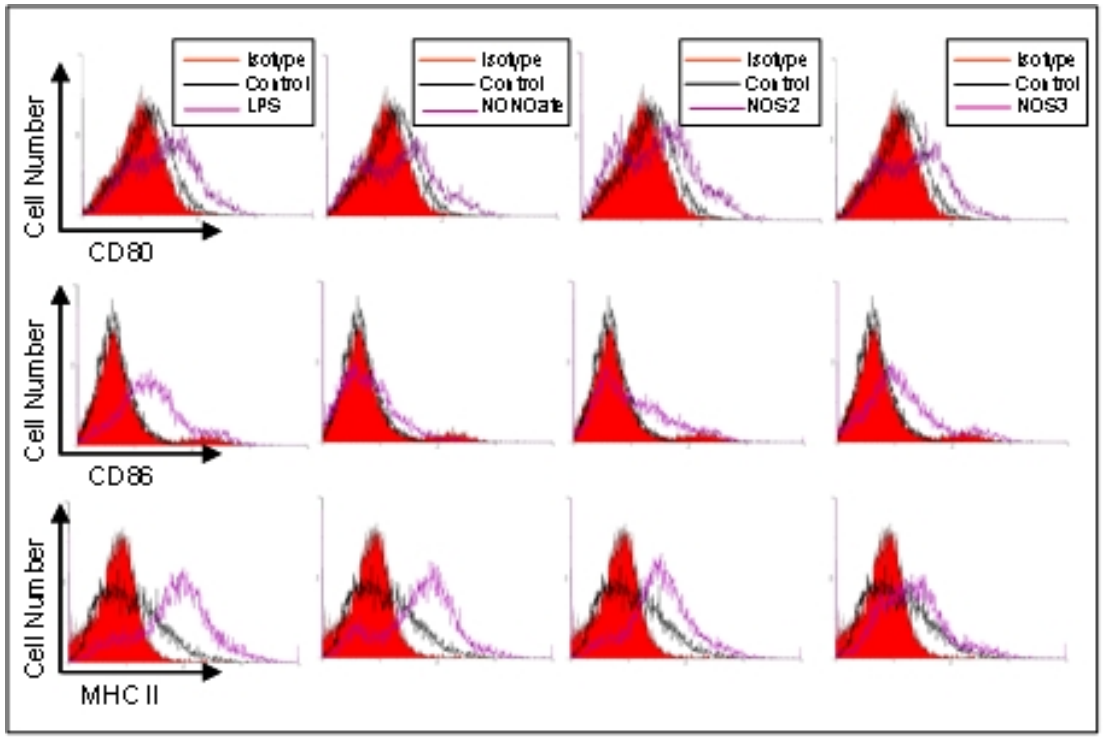


Figure 29: NO production enhances cell surface markers of DCs

Immature DCs were cultured in a medium only (Control), or a culture medium containing LPS or NONOate, or were transfected with mRNA encoding either NOS2 or NOS3. Cells were stained with CD80-FITC (upper panel), CD86-FITC (middle panel) and MHC class II-FITC antibodies (lower panel), followed by an analysis with flow cytometry. Control staining (red histograms) was performed with the corresponding isotype IgG, Mouse IgG2a for MHC class II, and mouse IgG1 for CD80 and CD86.

have shown that proteases such as caspases are involved in the degradation of molecules localised to the endosomal membrane trafficking pathway in immature DCs. The endogenous caspase inhibitor, DC-CASPIC, has also been shown to prevent the degradation of membrane tracking-related protein syntaxin 8 (SYN8) (Figure 30A). In this pathway, the inhibition of caspase activity results in the up-regulation of NOS protein and NO synthesis. Therefore, it is possible that NO may involve in the regulation of membrane trafficking-related proteins. To test this hypothesis, total cell extracts from untreated DCs or DCs treated with LPS, caspase inhibitor (CI), or NO donor NONOate were separated by SDS-PAGE, and the expressions of various membrane tracking-related proteins including vti1a, vti1b, α-adaptin, γ-adaptin and syntaxin 8, as well as NOS2, were detected by corresponding antibodies in a Western blot. Both caspase inhibitor (Figure 30B, *lane 3*) NO (Figure 30B, *lane 4*), as well as LPS treatment (Figure 30B, *lane 2*), increased the expressions of these proteins compared with untreated DCs. These results indicate that NO possibly has a similar effect to LPS in the regulation of membrane trafficking during the maturation of DCs.

4.2.1.4 NO enhances antigen presentation capability of DCs

Other than MHC class II, both the co-stimulatory molecules CD80 and CD86 were also up-regulated upon NONOate treatment. In addition, the NONOate-treated cells also displayed a similar pattern as mature DCs in the expression levels of many proteins. We thus hypothesised that NONOate alone

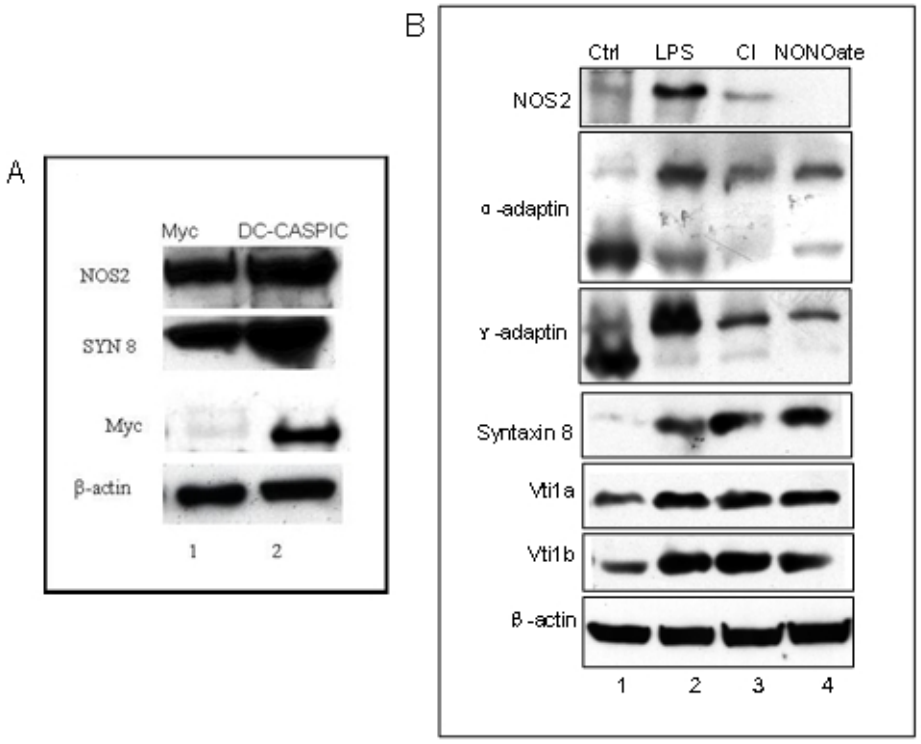


Figure 30: NO up-regulates endosomal proteins in DCs

(A) DC2.4 cells were lysates and probed with myc, syntaxin 8 (SYN8), NOS2 and *β*-actin antibodies in a Western blot. (B) Immature DCs were cultured in a medium alone (Ctrl), or a culture medium containing NONOate (NONOate) or caspase inhibitor (CI). Protein extracts derived from various treatments were separated by SDS-PAGE and transferred to nitrocellulose membrane. NOS2, *α*-adaptin, *γ*-adaptin, syntaxin 8, vti1a, vti1b and *β*-actin were detected using corresponding antibodies.

would be able to enhance antigen presentation in DCs in the absence of a general maturation signal such as LPS. To test this hypothesis, immature DCs were either untreated or treated with NONOate or LPS (as a positive control) prior to incubation with thymocytes in a mixed lymphocyte reaction assay for the detection of DC-induced T cell proliferation. Interestingly, DCs treated with NONOate alone were able to enhance T cell proliferation, although the enhancement was slightly weaker than that observed in LPS-treated DCs (Figure 31).

4.2.2 Nitric oxide inhibition of CD74 degradation

4.2.2.1 NO regulates degradation of CD74 in DCs

Owing to the fact that NO can induce a higher cell surface expression of MHC class II in DCs, it would be interesting to determine whether NO could also regulate the expression of protein molecules essential for the stability and intracellular trafficking of MHC class II complex in DCs. Day five immature DCs were either untreated (control) or treated with NONOate for 12 hours at 37 °C. Protein extracts were then separated by SDS-PAGE and probed with antibody against CD74 which is essential for MHC class II trafficking and antigen presentation in APCs (Matza *et al.*, 2003). NONOate significantly increased the expression of CD74 at the protein level in a dose-dependent manner (Figure 32C), but not at the mRNA level as shown by reverse transcription-PCR (Figure 32B *lane 6*). LPS, which induced NOS2 expression at both the transcriptional and translational levels, also increased CD74 protein expression (Figure 32A *lane 3* and B *lane 3*).

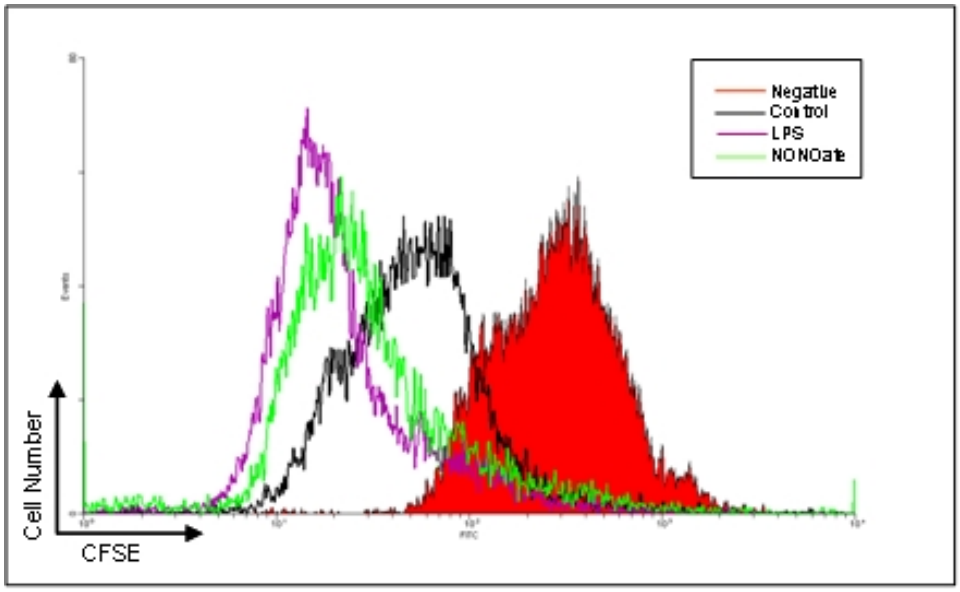


Figure 31: NO enhances the antigen presentation capability of DCs

CFSE-labelled T cells from BALB/c mice were co-cultured with DCs in a medium alone (control), or in a culture medium containing LPS or NONOate. T cell proliferation was determined by flow cytometry. The control labelled T cell (Negative) was performed similarly but in the absence of DCs

To further demonstrate the importance of NO in regulating the expression of CD74, immature DCs were either treated with L-NMMA (inhibitor of NOS1, NOS2 and NOS3 activities) alone, or treated with both L-NMMA and LPS. L-NMMA treatment alone did not vary the level of CD74 protein expression in immature DCs (Figure 32A *lane 2)*. However, LPS-induced CD74 expression was repressed by L-NMMA even in the presence of NOS2 (Figure 32A compare *lane 4* and *3*). Interestingly, we also observed that the expression of NOS2 was significantly increased in the presence of both LPS and L-NMMA (LPS / L-NMMA) as compared with LPS treatment, alone (Figure 32A *lane 3* and *4*). Unexpectedly, no increase in NOS2 at the transcriptional level was observed upon LPS/L-NMMA treatment as compared with LPS treatment alone (Figure 32B *lane 3* and *4*). Instead, a decrease in NOS2 mRNA synthesis was observed. This indicated the presence of positive feedback machinery where the inhibition of NOS activity by L-NMMA treatment enhanced the stability of NOS2 protein.

Based on our observations that both LPS and NONOate treatments up-regulate CD74 expression in DCs, we hypothesised that CD74 could be a substrate for caspases in immature DCs. Immature DCs were either untreated (control) or treated with 200 μM caspase inhibitor I (CI) for four hours at 37 °C. Protein extracts from either untreated or treated DCs were separated by SDS-PAGE and probed with antibody against CD74 in Western blot. Interestingly, both p31 and p41 isoforms of CD74 were significantly up-regulated in CI-treated DCs (Figure 32A, *lane 1* and *5*). This up-regulation of CD74 protein expression in CI-treated DCs was not due to the increase in the CD74 mRNA level (Figure 32B, *lane 5*). Furthermore, both LPS and CI treatment did not show significant changes to

CD74 expression in the mouse bone marrow-derived primary macrophages, suggesting that the effects of LPS and CI on the expression of CD74 are DC-specific (Figure 32A, *lanes 7–9*).

The other question to be answered was the specificity of NO. The results showed that NONOate exerts an effect on CD74 degradation at a concentration of as low as 1 μM. The effect becomes weak when the concentration is increased to 50 μM. This might be due to the toxicity of NONOate (Figure 32C).

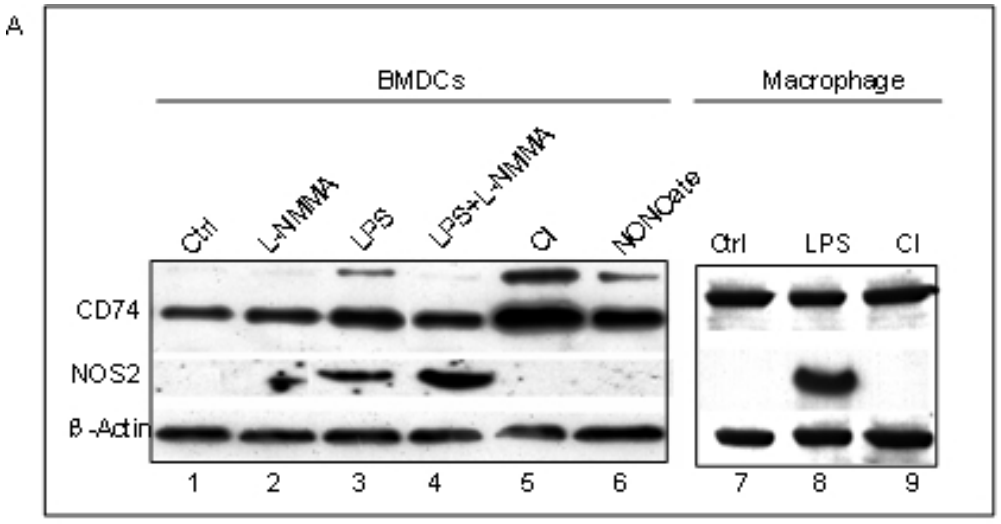
4.2.2.2 NO has similar effects as caspase inhibitor in regulating the degradation of CD74 protein in DCs

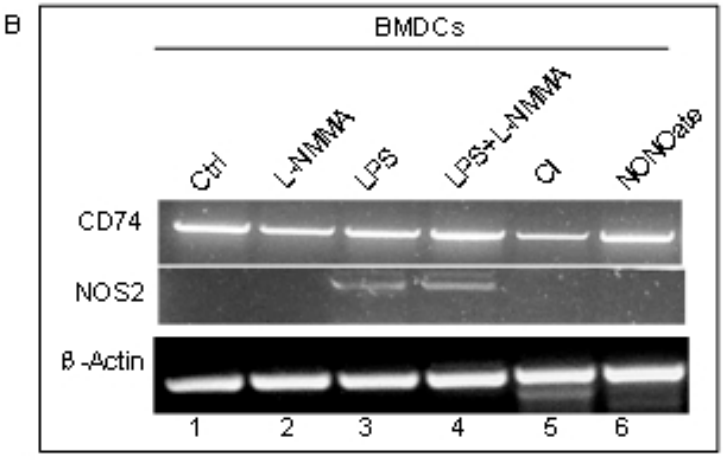
Since NO has similar effects as the caspase inhibitor in preventing the degradation of CD74, we next attempted to investigate whether NO directly inhibits caspase activity. The cells extracts from LPS-induced mature DCs or from immature DCs induced with NONOate were incubated with different caspase substrates (as indicated in Figure 33) for four hours. Our results showed that NONOate treatment inhibited around 50% of caspase activity towards most of the caspase substrates tested, except for IETD whose cleavage was approximately decreased around 30%. IETD is a substrate of caspase 8. However, in LPS-induced mature DCs, all caspase activities were decreased by half fold (Figure 33).

4.2.3 NOS is involved in the regulation of CD74 proteolytic degradation

4.2.3.1 Proteolytic degradation of CD74 is inhibited in NOS2-deficient DCs

In DCs, LPS induced NO production and NO acted as a caspase inhibitor in preventing CD74 degradation (Figure 33, 32A). Although NOS3 also


A
BMDCs
Macrophage
Ctrl
L-NMMA
LPS
LPS+L-NMMA
CI
NONOate
Ctrl
LPS
CI
CD74
NOS2
β-Actin
1
2
3
4
5
6
7
8
9


B
BMDCs
Ctrl
L-NMMA
LPS
LPS+L-NMMA
CI
NONOate
CD74
NOS2
β-Actin
1
2
3
4
5
6

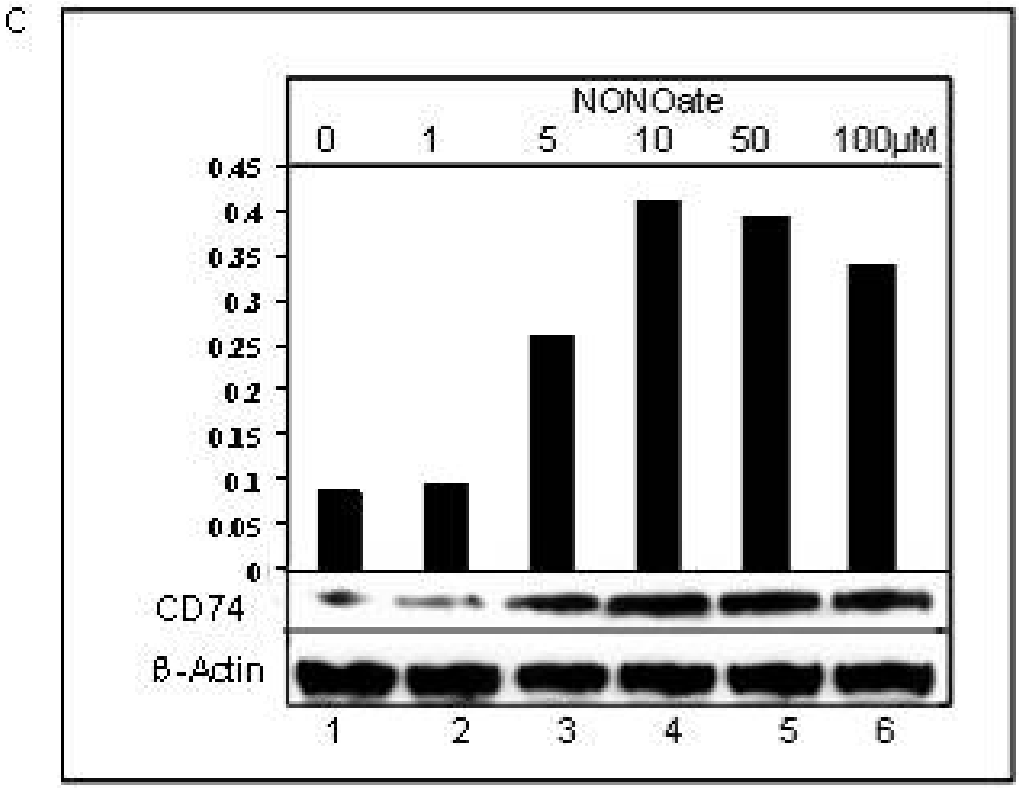


Figure 32: NO inhibits CD74 protein degradation

(A) (Left panel) Immature DCs derived from wild-type mice were either untreated (*lane 1*), or treated with L-NMMA (*lane 2*), LPS (*lane 3*), L-NMMA/LPS (*lane 4*), caspase inhibitor, CI (*lane 5*), or NONOate (*lane 6*). Protein extracts derived from various treatments were analysed by SDS-PAGE and Western blot. NOS2, CD74 and *β*-actin were detected by the corresponding antibodies (left panel). (Right panel) Macrophages were cultured in a medium alone (*lane 7*) or treated with either LPS (*lane 8*) or CI (*lane 9*). Cell extracts were separated by SDS-PAGE and analysed by Western blot. CD74 and *β*-actin were detected by the corresponding antibodies. (B) Total RNA was extracted from DCs treated as indicated in (A), and NOS2, CD74 and *β*-actin mRNA levels were determined by reverse transcription-PCR. (C) Immature DCs were treated with 1, 5, 10, 50 or 100 1μM NONOate for 16 hours. Cell extracts were subjected to SDS-PAGE and a Western blot analysis. CD74 and *β*-actin were detected by the corresponding antibodies. The bars represent the density of CD74 bands, which are normalised to *β*-actin bands

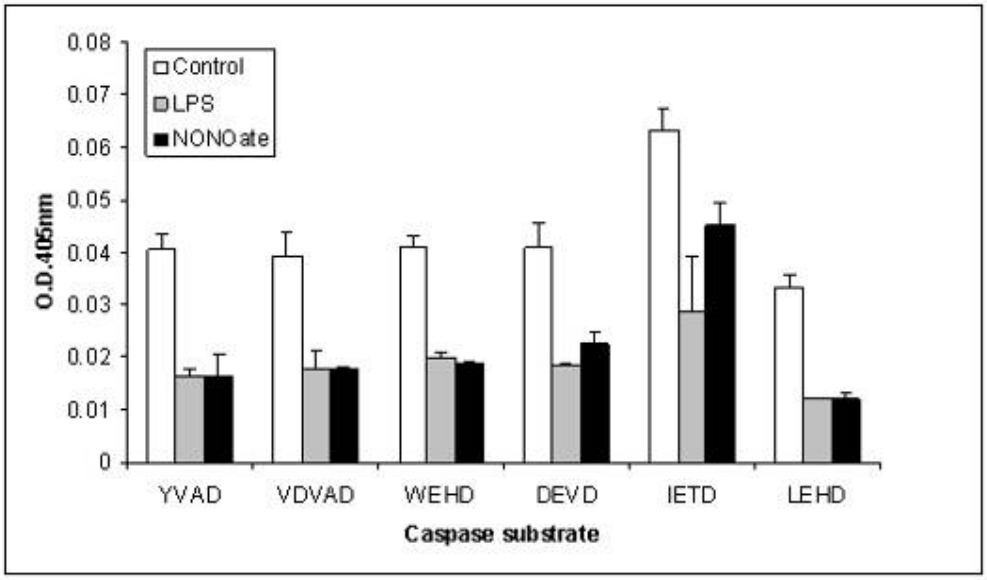


Figure 33: NO inhibits caspase activity

(A) Cell extracts from immature DCs, induced with either LPS (grey bar) or NONOate (black bar) were incubated with different caspase substrates (as indicated), respectively, for four hours. The caspase activities were then measured. Data shown were the mean ± *s.d.* from three independent experiments. ($p < 0.01$)

contributed to NO synthesis to a lesser extent, the LPS-induced NO synthesis in DCs was mainly catalysed by NOS2 (Figure 28A). To verify whether it was indeed NOS2 taking part in the regulation of CD74, the NOS2 gene knockout DCs were used as a model. The wild-type and $NOS2^{-/-}$ mice were either untreated or treated with LPS and the cell lysate were subjected to a Western blot analysis. CD74 protein expression was then detected by the anti-CD74 antibody. As expected, in immature wild type and $NOS2^{-/-}$ DCs, the CD74 protein was undetectable (Figure 34 *lane 1*and *3*). After induction with LPS, the CD74 proteins in wild type DCs increased significantly (Figure 34, *lanes 1* and *2*). The expression of CD74 protein was also up-regulated in mature $NOS2^{-/-}$ DCs, but the up-regulation was obviously compromised compared with wild type DCs. This result indicated that NOS2 participate in the regulation of CD74, although there might also be other NOS genes, such as NOS3, that are involved in this regulation.

4.2.3.2 NOS2 forms complexes with CD74

Nitric oxide is a labile free radical gas that functions as a signalling and effector molecule in controlling a variety of cellular functions. Because of its potent reactivity and diffusibility, NO production by NOS2 is expected to be stringently controlled to ensure target specificity. One way to achieve target specificity is by forming stable NOS2-targeted protein complexes. NOS had previously been reported to form complexes with intracellular proteins (Bogdan, 2001; Cao *et al*., 2001; Pritchard Jr *et al*., 2001). The interaction of NOS with specific heterologous proteins could be an effective mechanism to regulate the functions of these essential protein complexes. Based on our observations that CD74 was a substrate for caspases and NO could prevent the degradation of CD74 presumably via its ability to inhibit caspase activities, it would therefore be

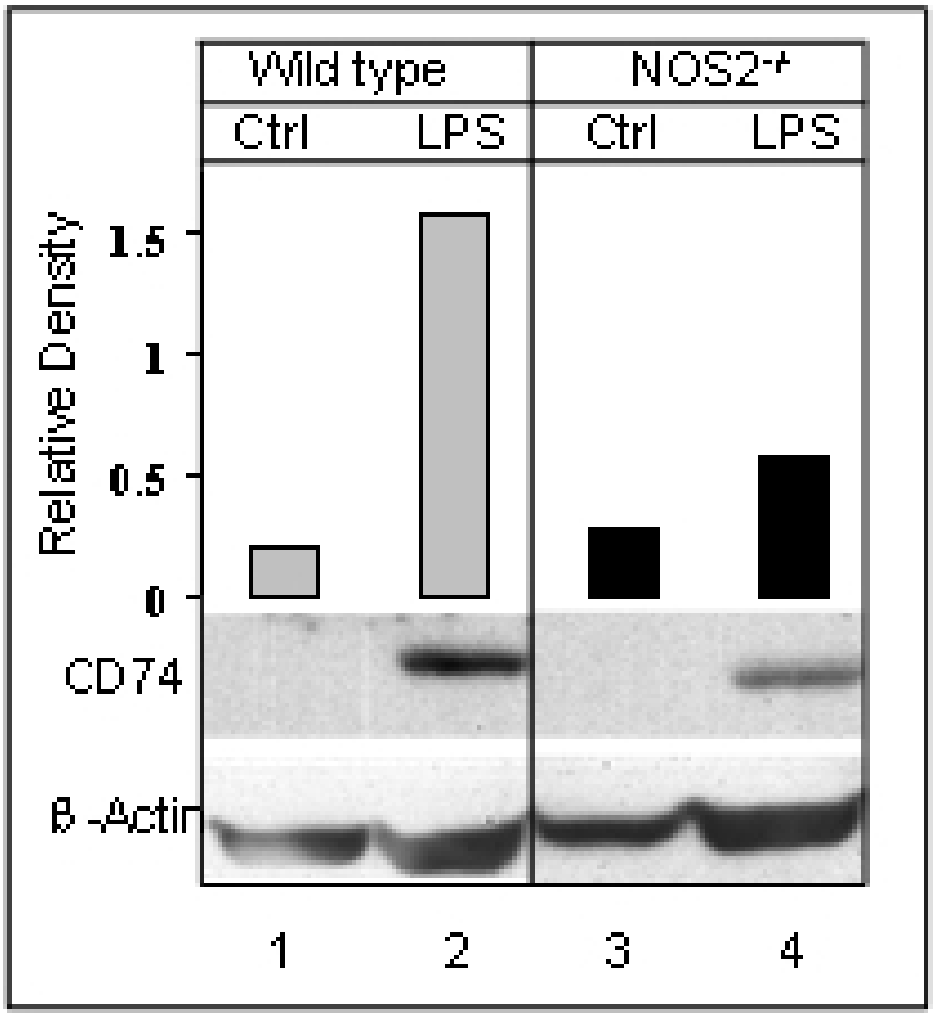


Figure 34: Proteolytic degradation of CD74 is enhanced in NOS2-deficient DCs

Immature DCs derived from the wild type (grey bar) and NOS2$^{-/-}$ (black bar) mice were either untreated (control) or treated with 1 μg/mL LPS. Cell extracts were separated by SDS-PAGE and analysed by a Western blot. CD74 and β-actin were detected by the corresponding antibodies. The bars are representative of the density of CD74 bands, which are normalised to β-actin bands.

interesting to determine whether NOS2, the key enzyme that catalyses the production of NO in maturing DCs, could form complexes with CD74, thus regulating CD74 degradation. First, we examined whether CD74 co-localises with NOS2 in maturing DCs. LPS-induced DCs were double-labelled with CD74 and NOS2-specific antibodies and analysed by confocal microscopy. CD74 partially co-localised with NOS2 to the endosomal-like (Figure 35A) and endoplasmic reticulum (ER)-like (Figure 35A, B and C) structures in mature DCs.

Next, we examined whether both NOS2 and CD74 could form a complex with each other. Co-immunoprecipitation studies using anti-NOS2 and anti-CD74 antibodies showed that NOS2 indeed formed a complex with CD74 (Figure 35D), and this was consistent with the earlier co-localisation study (Figure 35A).

4.2.4 Caspase 1 and caspase 11 are potentially involved in degradation of CD74 in DCs

Because DCs treated with CI expressed higher level of CD74 protein, we next made attempts to identify the caspases that potentially utilise CD74 as a substrate. DCs were either untreated or treated with peptide-based caspase inhibitors: Z-VAD-FMK (for caspases 1, 3, 4 and 7), Z-WHED-FMK (for caspases 1, 4, 5 [11 in mouse] and 8), Z-YVAD-FMK (for caspases 1 and 4), or Ac-DEVD-CMK (for caspases 3, 6, 7, 8 and 10). All of the inhibitors except Ac-DEVD-CMK elevated the CD74 protein level in DCs (Figure 36A). To determine the specificity of these inhibitors, DCs were incubated with various concentrations of either Z-WHED-FMK or Z-YVAD-FMK and then subjected to a Western blot analysis for the detection of CD74 protein expression. Although both inhibitors were able to inhibit CD74 degradation even at concentrations as low as 0.1 μM,

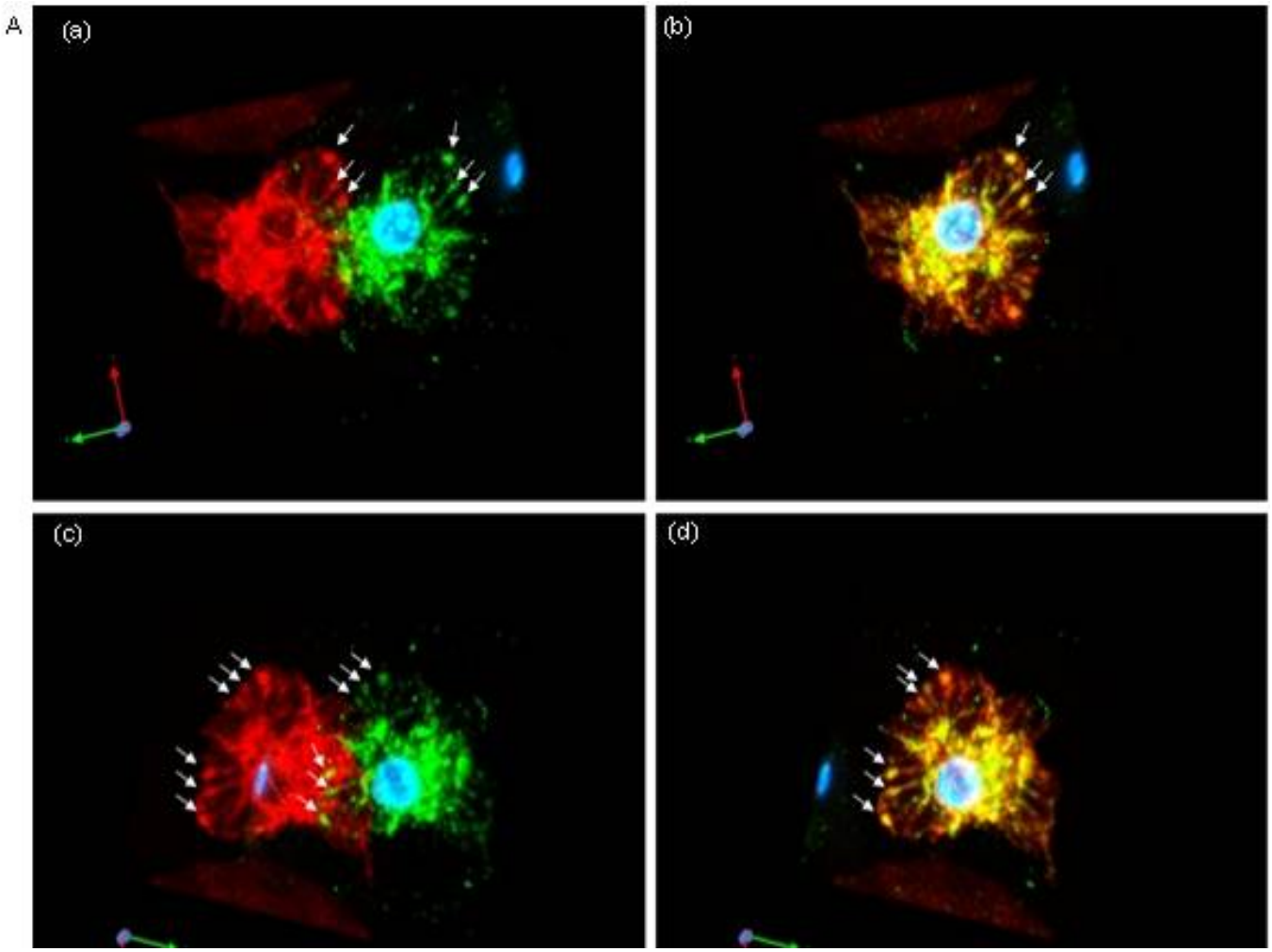
the inhibitory effect of Z-YVAD-FMK was relatively more specific than Z-WHED-FMK, based on its ability to inhibit CD74 degradation in a dose-dependent manner (Figure 36B). Due to the fact that Z-YVAD-FMK and Z-WHED-FMK are strong inhibitors for both caspase 1 and caspase 11, it is highly possible that CD74 could be a substrate for either caspase 1 and/or caspase 11.

Next, we investigated if both caspase 1 and 11 can form complexes with CD74. Co-immunoprecipitation studies using anti-NOS2 and anti-CD74 antibodies showed that caspase 1 and 4 indeed formed complexes with CD74 (Figure 36C). This further indicates that caspase 1 and/or 11 directly cleaves to CD74.

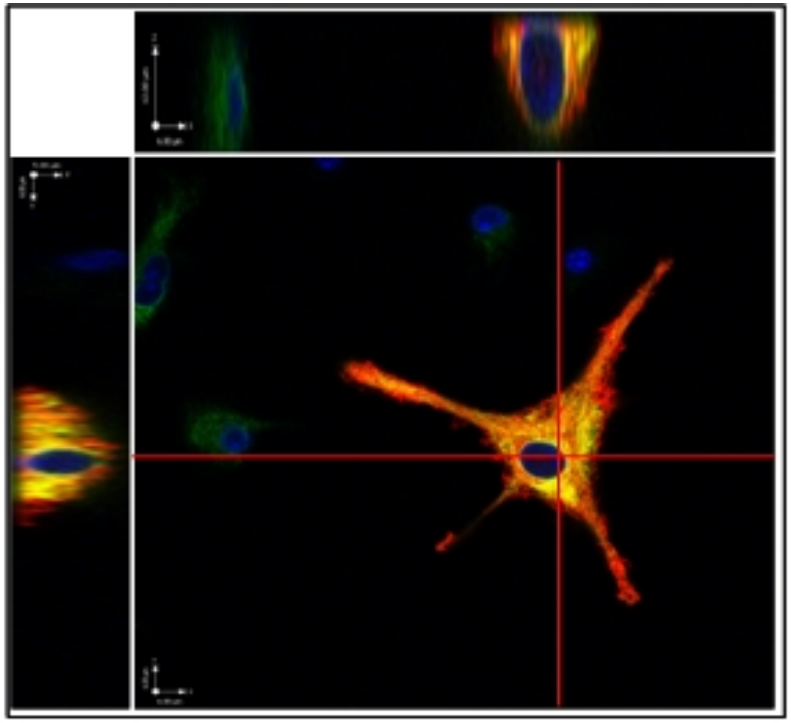
4.2.5 N-terminus of CD74 contains a caspase cleavage site

The screening of the amino acid sequence of the N-terminus of CD74 revealed a putative caspase recognition/cleavage site “DQRD”. This cleavage site is conserved in various mammalian animals, including the rat, human, pig and cow (Figure 37A).

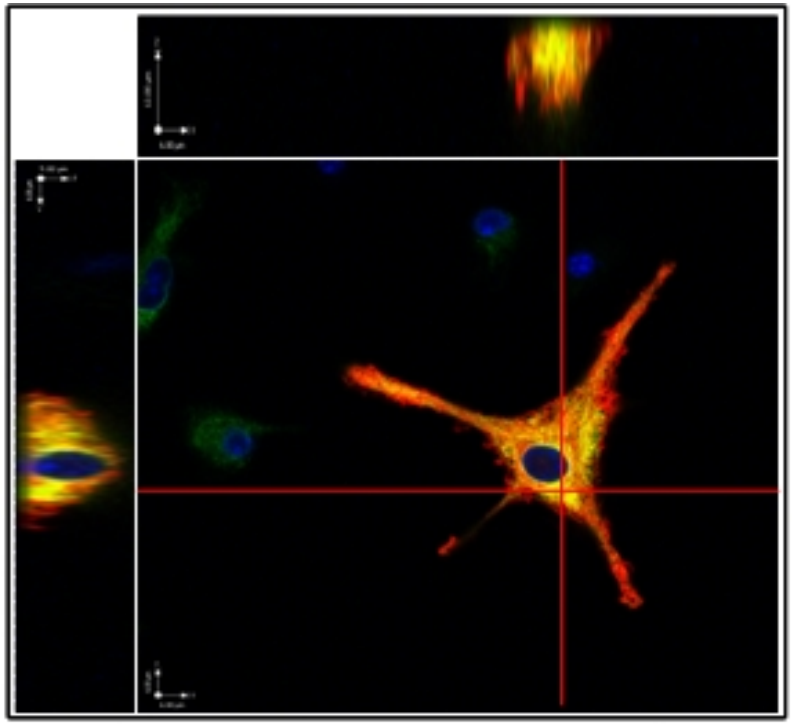
Next, we investigated whether the DQRD motif was important for the intracellular localisation of CD74. Myc-CD74 (wild-type) and Myc-(D–A)CD74 (D–A mutant) were expressed in DC2.4 cells and were detected using a specific antibody against c-Myc. Interestingly, a changing of the sequence “DQRD” motif to “DQRA” by point mutation did not cause a mis-localisation of CD74 in DCs (Figure 37B).


A
(a)
(b)
(c)
(d)

B



C



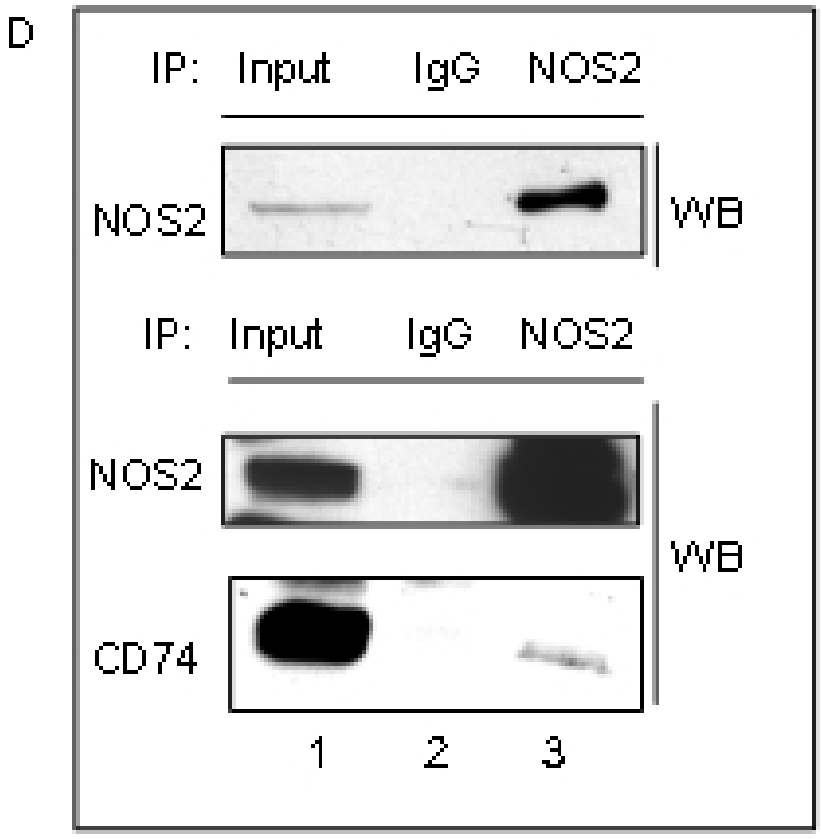
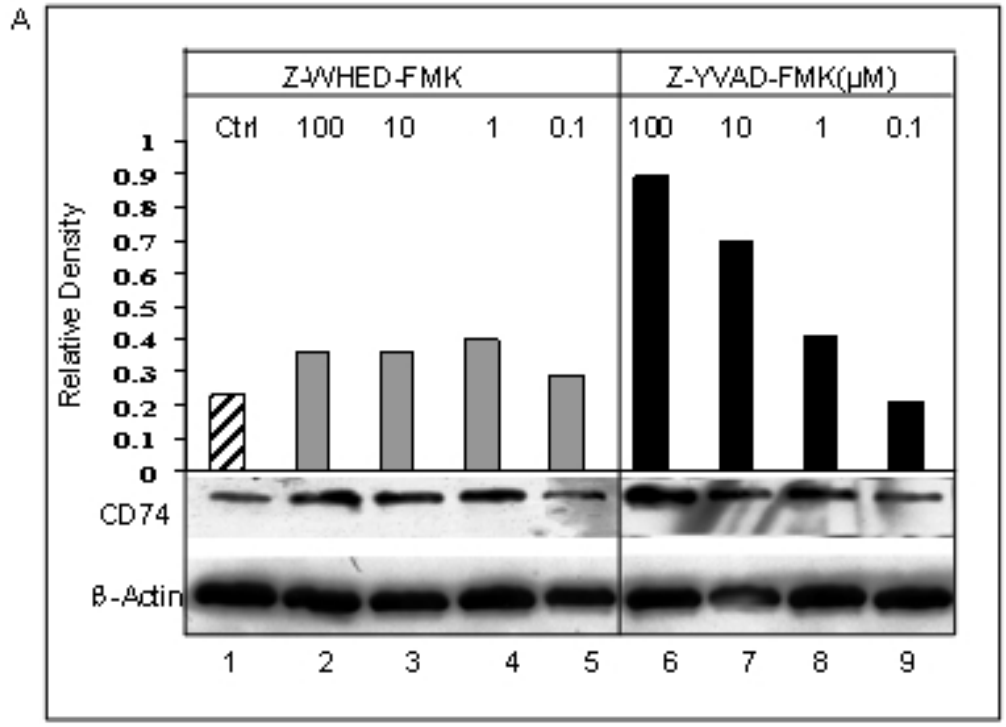
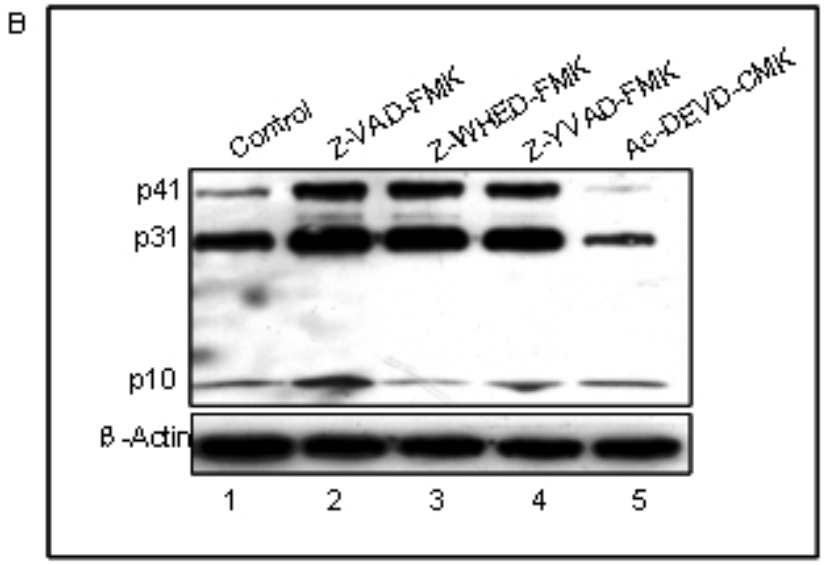


Figure 35: NOS2 forms complexes with CD74

(A) Stereo 3D rendering images of NOS2 and CD74 subcellular localisation in DCs. Co-localisation of NOS2 (red) with CD74 (green) was viewed from two different angles. Z-scaling=0.49μm, Z-stack size=12.6μm, number of slices=26. (B–C) X-Z/Y-Z projection of collected confocal images showing co-localisation of NOS2 with CD74 from two different positions. Z-scaling=0.43μm, Z-stack size=14.18μm, number of slices=34. Stereo 3D rendering and X-Z/Y-Z projection were performed using the Volocity visualisation software from Improvision. (D) Protein extracts derived from LPS-treated DCs were immunoprecipitated with CD74 or NOS2 antibodies as indicated and sequentially probed with NOS2 and CD74 antibodies as indicated, by a Western blot (IP: immunoprecipitation; WB: Western blot analysis).


A
Z-WHED-FMK
Z-YVAD-FMK(μM)
Ctrl
100
10
1
0.1
100
10
1
0.1
Relative Density
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
CD74
β-Actin
1
2
3
4
5
6
7
8
9


B
Control
Z-VAD-FMK
Z-WHED-FMK
Z-YVAD-FMK
Ac-DEVD-CMK
p41
p31
p10
β-Actin
1
2
3
4
5

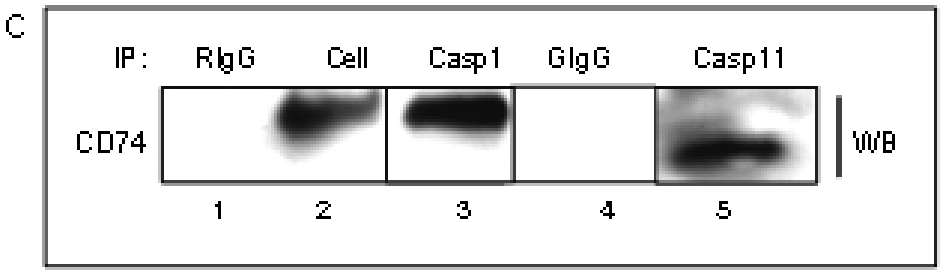


Figure 36: Caspases are involved in the degradation of CD74 in DCs

(A) Immature DCs were either untreated (control) or treated with several of 200 mM caspase inhibitors): Z-VAD-FMK, Z-WHED-FMK, Z-YVAD-FMK and Z-DEVD-CMK. Cell extracts were separated by SDS-PAGE and transferred onto nitrocellulose membrane. CD74 and β-actin were detected by the corresponding antibodies. (B) Immature DCs were either untreated (Ctrl) or treated for four hours with various concentrations (100, 10, 1, 0.1 μM) of Z-WHED-FMK or Z-YVAD-FMK, and the cell lysates were subjected to a Western blot analysis for CD74 protein expression. CD74 and β-actin were detected with corresponding antibodies. Bars represent the densities of CD74 bands (normalised to β-actin bands). (C) Protein extracts derived from LPS-treated DCs were immunoprecipitated with caspase 1, 11, or NOS2 antibodies as indicated and sequentially probed with caspase antibodies, as indicated in the Western blot analysis. (RIgG: rabbit IgG isotype control, cell: input, Casp1: caspase 1 antibody, GIgG: goat IgG isotype control, Casp11: caspase 11 antibody; IP: immunoprecipitation; WB: Western blot analysis)

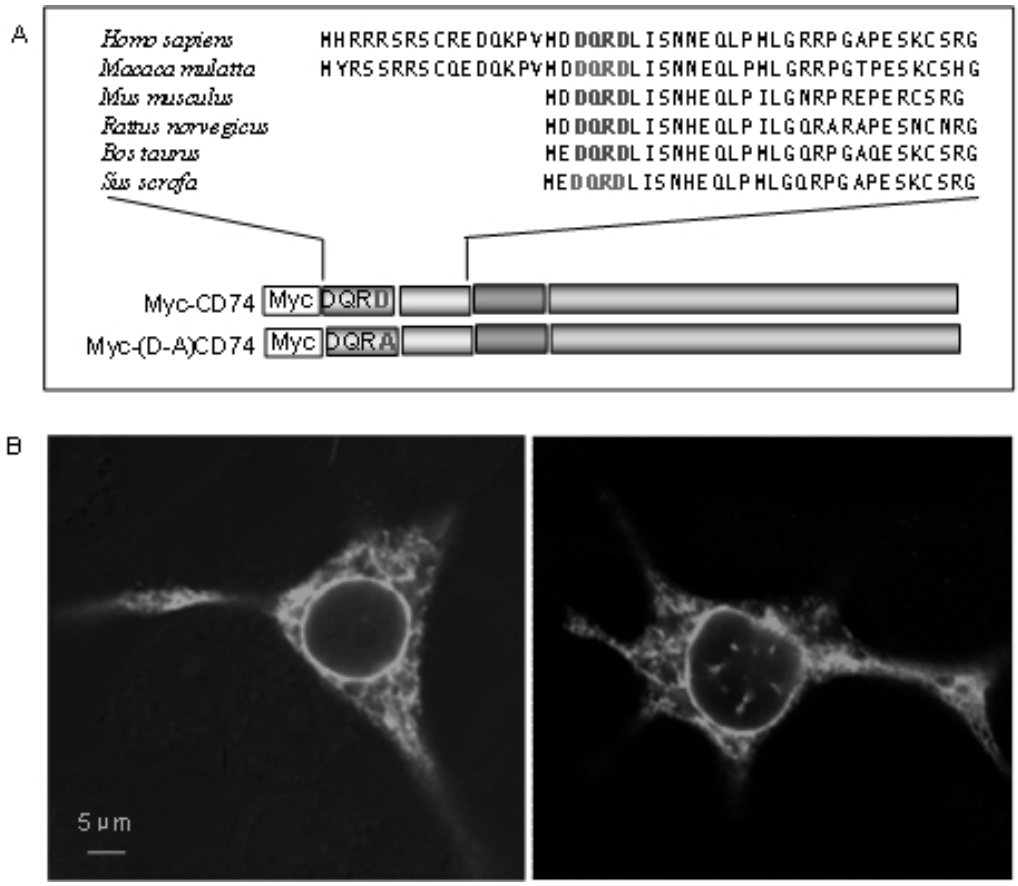


Figure 37: Caspase cleavage site on N-terminus of CD74

(A) The amino acid sequences of the CD74 cytosolic domain from different animals were aligned. The red font indicates the conserved caspase cleavage site (upper panel). (B) DC2.4 expressing either Myc-CD74 (right panel) or Myc-(D–A) CD74 (left panel) were fixed and stained with antibodies against c-myc, followed by the FITC-conjugated anti-mouse IgG. Images were taken using the Olympus Fluoview 500 at 1000 × magnification.

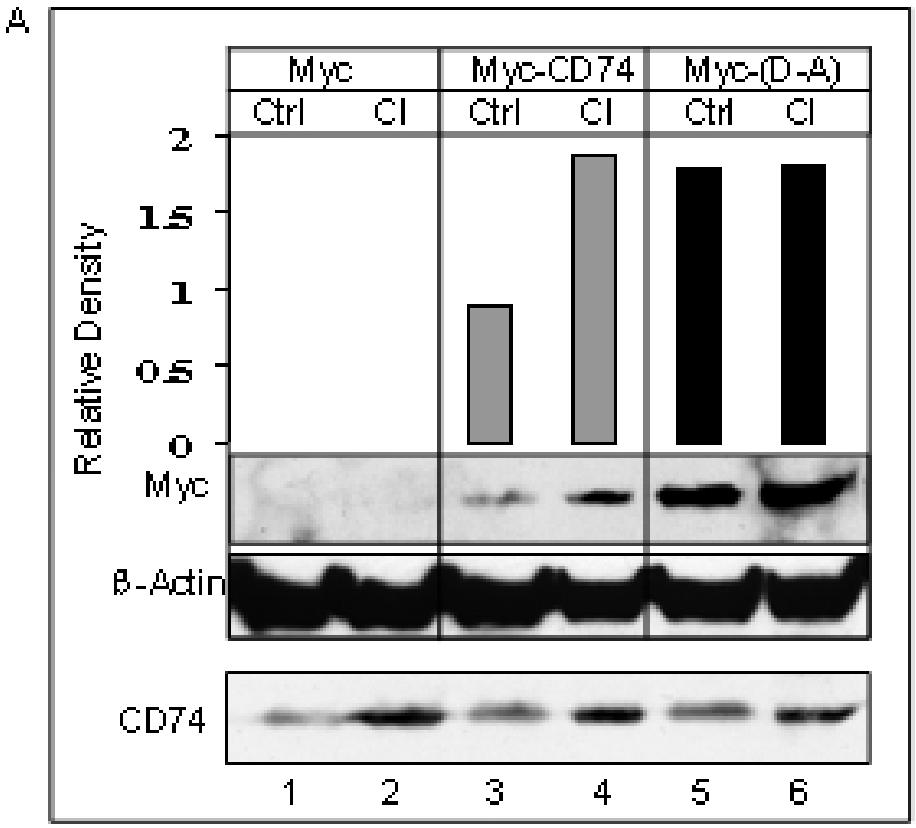
4.2.6 "DQRD" motif is a caspase recognition and cleavage site

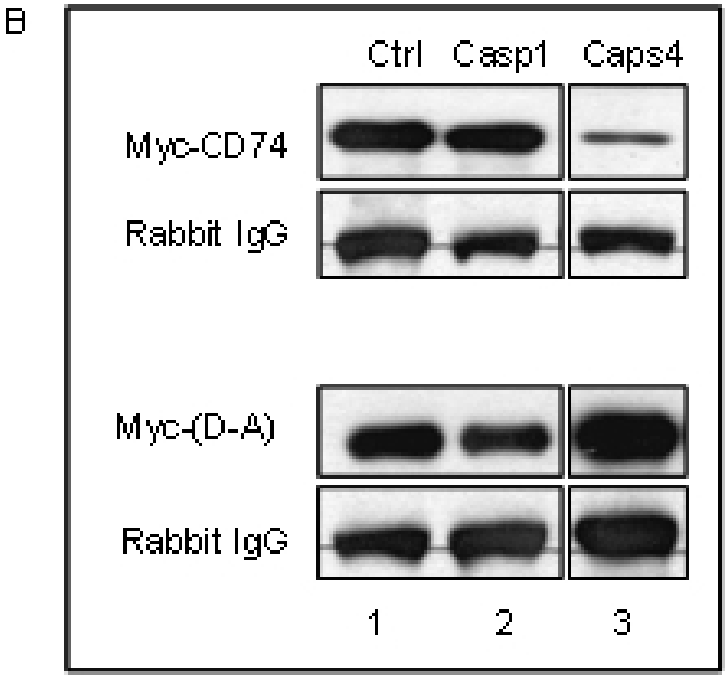
To verify whether the "DQRD" motif was a caspase recognition and cleavage site, protein extracts derived from either untreated or CI-treated DCs [expressing either Myc-CD74 or Myc-(D–A)CD74,] were analysed by a Western blot analysis using antibodies against c-Myc and CD74. The degradation-inhibitory effect of CI was more drastic on Myc-CD74 than on Myc-(D–A)CD74 (Figure 38A, compare *lane 3/4* and *lane 5/6*). In addition, we consistently observed that the quantity of total Myc-(D–A)CD74 proteins was significantly higher than the amount of Myc-CD74 proteins in the untreated DCs. This strongly suggested that the "DQRD" motif was indeed a caspase cleavage site and that D–A mutation significantly increased the stability of CD74 in DCs. As expected, CI-treatment increased the expression of endogenous CD74 as detected by IN–1 monoclonal antibody (Figure 38A). To further confirm that CD74 was a substrate for caspases and that the "DQRD" motif on the N-terminus of CD74 was a caspase cleavage site, Myc-CD74 or Myc-(D–A)CD74 was expressed in DC2.4, immunoprecipitated using CD74-specific monoclonal antibody (IN–1) and digested with recombinant caspase 1 and caspase 4 *in vitro* prior to detection, using an anti-Myc polyclonal antibody. Immunoprecipitated Myc-CD74 but not Myc-(D–A) CD74 was preferentially cleaved by caspase 4 (Figure 38B, *lane 3*). On the other hand, Myc-CD74 was not cleaved by caspase 1 (Figure 38B, *lane 2*). However, it was worth noting that a small amount of Myc-(D–A)CD74 was cleaved by caspase 1 (Figure 38B, *lane 2*, lower panel). The reason for this was unclear, but there was a possibility that the D–A mutation on the "DQRD" motif might have created a site more receptive to caspase 1 activity. In addition, the "DQRD" motif was shown to be essential for the NOS2-CD74 interaction based

on the observation that wild-type Myc-CD74 but not the mutant Myc-(D–A)CD74 interacted with NOS2 (Figure 38C).

4.2.7 Over-expression of CD74 enhances MHC class II, CD80 and CD86 cell surface expression in immature DCs

The importance of CD74 in MHC class II trafficking and antigen presentation has previously been reported (Matza *et al.*, 2003). Based on the observations that NO treatment increased the expression of total CD74 protein and several essential endosomal protein molecules and at the same time up-regulated the cell surface expression of MHC class II, CD80 and CD86 in DCs, it was of great interest to determine whether an over-expression of CD74 alone could cause an increase in the cell surface expression of MHC class II and the two essential co-stimulatory molecules CD80 and CD86 in immature DCs. Our results showed that an over-expression of either wild type (Myc-CD74) or D–A mutant CD74 (Myc-(D–A)CD74) by mRNA electroporation enhanced the cell surface expression of MHC class II, CD80 and CD86 in immature DCs (Figure 39). Enhanced CD74 expression alone could therefore result in an increase of MHC class II, CD80 and CD86 expression on the surface of immature DCs.


A
Myc
Myc-CD74
Myc-(D-A)
Ctrl
CI
Ctrl
CI
Ctrl
CI
Relative Density
2
1.5
1
0.5
0
Myc
β-Actin
CD74
1
2
3
4
5
6


B
Ctrl
Casp1
Caps4
Myc-CD74
Rabbit IgG
Myc-(D-A)
Rabbit IgG
1
2
3

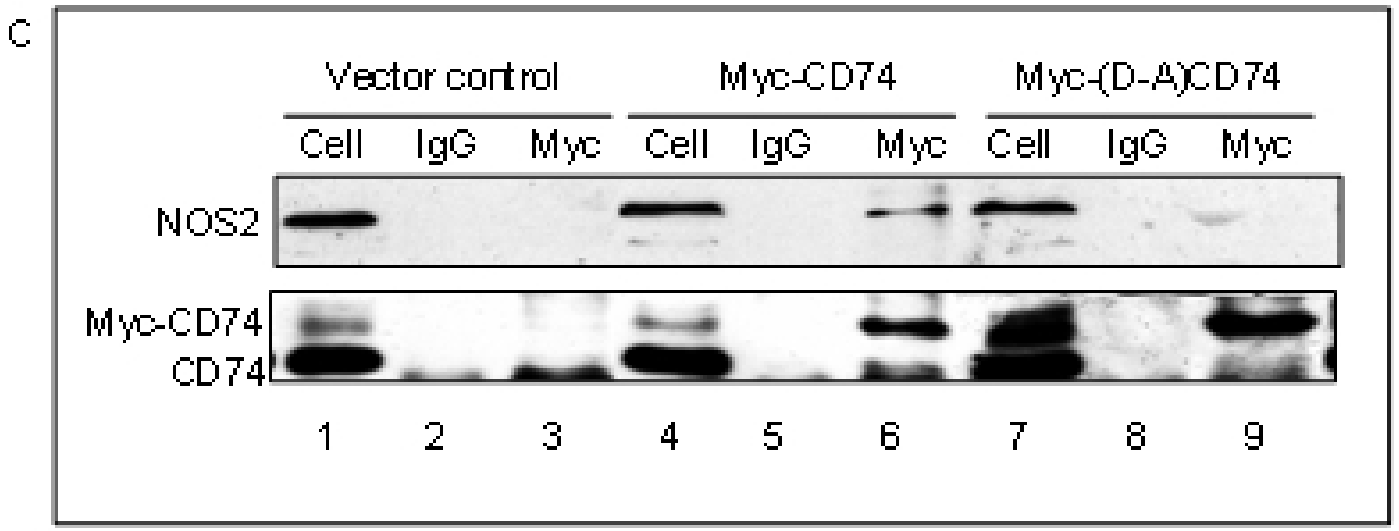


Figure 38: "DQRD" motif - a caspase recognition and cleavage site

(A) DC2.4 cell extracts derived from either untreated or CI-treated DCs [expressing either Myc-CD74 or Myc-(D–A)CD74] were analysed by a Western blot, using antibodies against c-myc and CD74. Bars represent the density of myc bands normalised to β-actin bands. (B) Myc-CD74 and Myc-(D-A)CD74 were expressed in DC2.4, immunoprecipitated using CD74-specific mAb (IN-1), and digested with recombinant caspase 1 and caspase 4 *in vitro* prior to detection using anti-myc and anti-rabbit IgG polyclonal antibodies. (C) DC2.4 were transfected with Myc-CD74 or Myc-(D–A)CD74 followed by induction with LPS. Cells were harvested, immunoprecipitated with anti-c-myc antibody, and probed with NOS2 and CD74 antibodies in a Western blot. The lysates were also precipitated with the corresponding isotype IgG.

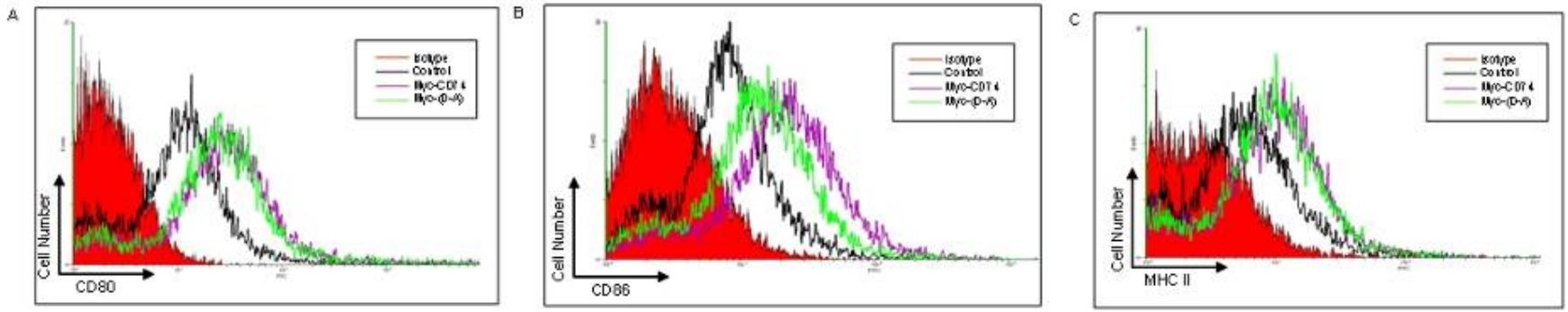


Figure 39: Over-expression of CD74 increases MHC class II cell surface expression

(A-C) DCs were transfected (by electroporation) with luciferase (solid line), Myc-CD74 (purple line) or Myc-(D–A)CD74 (green line) mRNA. After a 24-hour incubation, cells were harvested and double-labelled with antibodies against CD80-FITC (A), CD86-FITC (B) or MHC class II-FITC (C), followed by flow cytometric analysis.

4.3 Discussion

4.3.1 NO partially promotes maturation of DCs

Our studies in chapter 3 revealed that DC-CASPIC is one of the key molecules that regulate nitric oxide (NO) synthesis and thereby antigen presentation during maturation of DCs. In this chapter, I dissected the detailed mechanism of NOS/NO-enhanced antigen presentation during maturation of DCs. NO is a free-radical gas molecule that is implicated in a large number of fundamental cellular processes, such as vascular regulation, neurotransmission and immunity (Bogdan, 2001; Maniscalco *et al.*, 2007). NO reacts in water with oxygen and its reactive intermediates yield (i) stable anions such as nitrite (NO_2^-) and nitrate (NO_3^-), (ii) unstable higher oxides such as nitrogen trioxide (N_2O_3), and (iii) unstable peroxides, peroxynitrite ($ONOO^-$). In this study, our results show that NO can partially initiate maturation of DCs. Firstly, NO has the ability to induce the cell surface expression of MHC class II, CD80 and CD86 all of which are hallmarks of DCs going through maturation. In human monocytes, NO is reported to be involved in the acquisition of a more activated phenotype of DCs such as CD1a, CD80 and MHC class II (Fernandez-Ruiz *et al.*, 2004). Moreover, the *N*-nitroarginine, an NO synthase inhibitor, produces a reversion of the phenotype of human mature DCs, induced by anion superoxide anion (Isenberg, 2003). Secondly, similar to the effect of LPS (Wong *et al.*, 2004; Santambrogio *et al.*, 2005), No-donor NONOate treatment also causes an up-regulation of several essential molecules in the endosomal membrane trafficking pathways in DCs. Thirdly, NO-induced DCs acquire the capability to stimulate and enhance T cell proliferation *in vitro*. However, there are several reports showing that the NO/NO

donor, SNAP, or IFN-*γ*, inhibits alloantigenic T cell proliferation (Lu *et al.*, 1996d; Katagiri *et al.*, 2000). This inconsistency may be due to the different protocols used. In these studies, the NO donor was directly added into the DCs and T cell mixture, whereas in our study, the NO or NO donor was washed away before incubating with the T cells. Another reason may be that different types of the NO donor have been used, and thus different levels of NO may have been produced. A higher concentration of NO always accompanies cell apoptosis, whereas low concentrations of NO are able to selectively enhance the differentiation of helper T cells (Th_1) (Niedbala *et al.*, 2002).

4.3.2 NOS2-CD74 partnership is essential to keep CD74 intact

In LPS-induced DCs, the production and secretion of the endogenous NO correlates with NOS2 expression (Bonham *et al.*, 1996). NO is a labile free radical molecule, which does not act through a receptor, but instead enters cells indiscriminately (Coleman, 2001). Therefore, the specificity of its action fully depends on its concentration and its proximity to target molecules. For NO to effectively exert its inhibitory actions on caspases via *S*-nitrosylation to protect proteins/complexes from proteolytic degradation, it is essential for it to be present in high, localised concentrations. Hence, it is critical for NOS to localise closely with proximity to target proteins/complexes. Interactions between NOS and several intracellular proteins have recently been reported by several laboratories (Cao and Eldred, 2001; Lo *et al.*, 2001; Bogdan, 2001b). One of these reports has suggested that that interaction of NOS2 with the heat shock protein enhances the gp96-dependent production of NO in immature DCs (Panjwani *et al.*, 2002). Our study shows that NOS2 partially co-localises, and more interestingly, interacts

with CD74 in DCs. Thus, NOS2, which is in proximity to CD74, could catalyse the production of sufficiently high, localised concentrations of NO to protect CD74, as well as other endosomal membrane trafficking related proteins, from caspase-dependent degradation.

Furthermore, point mutation and co-immunoprecipitation studies revealed that the association of NOS2 and CD74 is dependent on the "DQRD" motif on the cytosolic domain of CD74. However, LPS-induced NOS2 without NO synthesis (in the presence of $_{L}$-NMMA that inhibits the activity of all three forms of NOS) did not reduce CD74 degradation. Thus, the possibility that NOS2 binding to CD74 might have prevented caspases from accessing the recognition/cleavage site ("DQRD") on the N-terminus of CD74, by inducing either steric hindrance or conformational change, is excluded.

Although the "DQRD" motif is essential for both recognition/cleavage by caspases and for the interaction between of NOS2 and CD74, there is a possibility that the binding of caspases to the N-terminus of CD74 is independent of NOS2 binding. In immature DCs where the expression of NOS2 is low, caspase binds to the "DQRD" motif and cleaves CD74 efficiently. However, upon induction by LPS that initiates maturation of DCs, the expression of NOS2 increases. NOS2 then binds to the N-terminus of CD74 in proximity to the "DQRD" motif. Although this binding of NOS2 does not interfere with the binding of caspases to the "DQRD" motif, it can catalyse the production of NO, which inhibits caspases and prevents CD74 degradation. Thus, the NOS2-CD74 partnership is essential to keep CD74 or its complexes intact. In fact, it is of great importance for CD74 to bind to NOS2 immediately after synthesis and to be inserted into the ER

membrane. This is consistent with our observation that NOS2 co-localises with CD74 on the ER-like membrane structures in maturing DCs. Although NOS2 is a cytosolic protein, it has been reported that NOS2 trafficks with vesiculotubular structures to dendritic clusters in hippocampal neurons (Wu *et al.*, 2000). Moreover, NOS2 is also able to bind to several membrane proteins, such as NAP-110 and kalirin in which the functional significance of these interactions has not yet been established (Navarro-Lerida *et al.*, 2006).

4.3.3 Both NOS2 and NOS3 are involved in the regulation of CD74

The protein expression level of CD74 was low in both the wild type ($NOS2^{+/+}$) and $NOS2^{-/-}$ immature DCs. As expected, after treatment with LPS, the expression of CD74 in wild type DCs increased dramatically. Interestingly, we also observed an increase of CD74 expression in $NOS2^{-/-}$ DCs after LPS induction, suggesting that other LPS-inducible forms of NOS might also be involved in protecting CD74 from degradation during maturation of DCs. In this case, NOS3 could be the likely candidate based on the following reasons: (i) Currently, there are three known forms of NOS (NOS1, NOS2 and NOS3) of which only NOS2 and NOS3 have been shown to be expressed in non-neuronal cells (Hickey *et al.*, 1997); the results show that DCs expressed NOS3 and the expression of NOS3 was inducible by LPS; (ii) The activity of NOS2 is partially dependent on the expression of NOS3 (Vo *et al.*, 2005; Connelly *et al.*, 2005).

4.3.4 CD74 is one of the substrates of caspases

The MHC class II-associated invariant chain, CD74, was one of the molecules up-regulated in DCs after treatment with either LPS or NONOate.

CD74 is essential for proper MHC class II folding in the ER, and for targeting MHC class II-CD74 complex to the endosomes (Becker-Herman *et al*., 2005; Busch *et al*., 2005). In the endosomes, CD74 protein undergoes degradation via several stepwise proteolytic processes (executed by various specific proteases), forming cleavage products, p31, p22 and p10. The final protease cleavage product, CLIP (a product derived from the cleavage of p10), a short peptide of 23 amino acids, remains bound to the peptide-binding groove of the MHC class II-αβ complex. Every proteolytic step is executed by specific proteases and the cleavage of proteins could be one of the mechanisms employed by cells to regulate protein expression levels at various stages of cell development. During maturation of DCs, the activities of proteases such as caspases are repressed, resulting in the accumulation of a number of endosomal proteins (Wong *et al*., 2004). In conjunction with this, the full-length CD74 protein (p31) is also up-regulated upon DC maturation (Engering *et al*., 1998; Gomez *et al*., 2004 and this study). An unaltered CD74 mRNA level in DCs after treatment with LPS excludes the possibility of up-regulation at the transcriptional level. Although several major proteases involved in the terminal stages of CD74 processing have been identified, very little is known about how the proteolytic degradation of CD74 was regulated in maturing DCs. A previous report has suggested that asparagine endopeptidase (AEP) was involved in the degradation of CD74 protein in B cells (Manoury *et al*., 2003). However, no difference in the processing of the CD74 has been observed between AEP-deficient and wild type mice (Maehr *et al*., 2005). Our results strongly suggest that caspase could be one of the major proteases that cleaves/degrades CD74 based on the observations that (i) the inhibition of caspases activity by the caspase inhibitor (CI) results in the accumulation of CD74

proteins; (ii) CD74 coimmunoprecipitates with caspase 1 and caspase 4; and (iii) the immunoprecipitated Myc-CD74 protein is a cleavage substrate for caspases *in vitro*. A screening of the N-terminal cytoplasmic domain of CD74 identified one potential caspase recognition/cleavage site, "DQRD", which is conserved in humans, mice and rats. In addition, the motif "DQRD" has been shown to be a specific cleavage site for caspase 3 (Houde *et al*., 2003). In this study, however, a caspase 3-specific inhibitor was not able to inhibit the degradation of CD74 in DCs. Further experiments using recombinant caspase 1 and caspase 4 have shown that myc-CD74, but not myc-(D–A)CD74 is preferentially cleaved by recombinant caspase 4. Furthermore, the disruption of the "DQRD" motif by point mutation [myc-(D–A)CD74] significantly increases the half life of CD74, further supporting the hypothesis that the "DQRD" motif is a caspase recognition/cleavage site.

Previous studies have suggested that caspase activity in immature DCs is relatively high and that activity is significantly inhibited in mature DCs by a mechanism closely linked to the activity of NOS2. NOS2 catalyses the synthesis of NO, which may in turn *S*-nitrosylate the catalytic domain of active caspases (Rossig *et al*., 1999; Tannenbaum and White, 2006)**.** This is consistent with our observation that CD74 degradation is repressed by treating immature DCs with the NO donor (NONOate).

Our results show that CD74 is a substrate for caspases and that the N-terminus of CD74 contains a single caspase cleavage site. Cleavage at the N-terminus of CD74 by proteases is plausible based on the fact that it is the only part of the CD74 molecule that is exposed to the cytoplasm. Since CD74 degradation

is inhibited in the presence of caspase inhibitors, it is likely that cleavage at the N-terminus by caspases alone could initiate the degradation of CD74. In addition, based on the localisation of CD74 on the ER-like compartment in maturing DCs, there is a possibility that the degradation of CD74 could be initiated by caspase 4 on the cytoplasmic surface of the ER membrane in DCs. This is supported by previous reports that caspase 4 is one of the caspases inhibited during DC maturation and caspase 4 is localised to the ER membrane (Nakagawa *et al*., 2000; Wong *et al*., 2004; Hitomi *et al*., 2004; Kim *et al.*, 2006). Caspase 4 has been shown previously to be essential for cytokine maturation and inflammation, and it has been reported to play key roles in ER stress- and amyloid-β-induced cell death (Kamada *et al*., 1997; Hitomi *et al*., 2004). Although caspase 4 is shown here to cleave and presumably to initiate CD74 degradation in DCs, the mechanism that regulates such an event is still unclear. While our results indicate that the degradation of CD74 could be initiated on the ER membrane, based on the ER-localisation of caspase 4, we could not exclude the possibility that CD74 is degraded after being targeted to the endosomes. This is supported by the fact that an N-terminus deleted CD74 construct, which has the endosomal targeting dileucine motif restrained, is still able to target to the endosomes (data not shown). Further experiments along this line will shed more light on this issue.

4.3.5 Increased CD74 enhances the function of DCs

The protection of CD74 from degradation could be one of the mechanisms that contribute to the increase in antigen presentation efficiency in maturing DCs. Previous studies showed that both p31 and p41 forms of CD74 promote MHC class II antigen presentation in mouse fibroblast cells (Serwe *et al*., 1997). This is

consistent with our observation that the inhibition of CD74 degradation by either the exogenous NO or the over-expression of NOS2 accompanies an increase in MHC class II cell surface expression in DCs. In addition, the over-expression of CD74 by mRNA electroporation in DCs increases the cell surface expression of CD80/CD86 and MHC class II.

This further supports our model that NO is the key molecule that inhibits the caspase-dependent degradation of CD74 and other endosomal proteins during LPS-induced DC maturation. Controlling the half life of these proteins is thus vital in regulating the trafficking of the MHC class II complex and antigen presentation during DC maturation. It has been recently shown that proteolytic peptides derived from CD74 are able to modulate cell proliferation and survival (Starlets *et al*., 2006). Thus, the regulation of the CD74 half life by caspases could have contributed significantly to the signalling cascades that control cell proliferation and survival.

4.3.6 Conclusion

The post-translational regulation of CD74 plays an important role in MHC class II trafficking and antigen presentation in DCs. Nevertheless, the post-translational regulation of CD74 is rather complicated and has not yet been fully elucidated. Hence, the primary objective of this study is to investigate a new pathway of CD74 post-translational regulation. A novel CD74 regulatory pathway involving caspases and NO has been identified.

To determine whether caspases are involved in the post-translational regulation CD74, DCs were treated with various types of caspase inhibitors and

their effects on the expression of CD74 were examined. All of caspase inhibitors except the caspase 3 inhibitor up-regulated the CD74 protein. In addition, the co-immunoprecipitation of caspases 1 and 4 with CD74 indicates the interaction of CD74 with these caspases. The mutations of the caspase cleavage site from DQRD to DQRA, which lies on the N-terminus of CD74, diminished the accumulation of CD74. Consistent with our results, it was also found that the caspase activity decreased during the maturation of DCs. From these observations, a new pathway can be illustrated. During the maturation of DCs, the caspase activity decreases and results in the accumulation of its substrate CD74. The up-regulation of CD74 provides DCs with the antigen-presenting capability. In this study, although different types of caspase inhibitors have been employed, the results presumably exclude solely the possibility of caspase 3, because it would be rather difficult to monitor the inhibitors' specificity under the physiological conditions. Nevertheless, the involvement of caspases in CD74 post-translational regulation as reported in this study has undoubtedly opened a new area in immunoregulation, because caspases have long been thought to act primarily as inducers of apoptosis. On the other hand, there are also other CD74 regulation pathways in DCs, such as the cathepsin-mediated pathway. It would therefore be interesting to investigate the relationship between the caspase-mediated pathway and the other regulatory pathways in CD74 regulation. DCs from caspase knockout mice would undoubtedly provide useful information. The knockout studies would also help to identify the specific caspases involved in the pathway.

In this study, factors at the upstream of caspase-mediated CD74 regulation pathway have also been investigated. The exogenous NO donor, NONOate, has a similar effect on on the accumulation of CD74 as caspases in DCs. This may be

due to the *S*-nitrosylation of caspases by NO [Rossig L, 1999]. In DCs, the production and secretion of endogenous NO are induced by LPS and are mainly correlated with NOS2 expression. However, too much NO is undesirable because NO is a labile free-radical molecule, which may cause damage to the cells, and at the same time, it is an effector molecule controlling a great variety of physiological functions in DCs such as cell migration, cytokine production as well as apoptosis [Giordano D,2006, Lu L, Bonham, 1996]. Therefore, NO level and thus the activity of NOS2 must be tightly regulated in DCs. Our data show that in DCs, NOS2 is present in the same intracellular compartment as CD74, such that NO will be produced adjacent to CD74 molecules. Therefore, NO could attain a gradient concentration sufficient to protect CD74 from caspase cleavage, but with limited undesirable effects. Furthermore, our mutagenesis and immunoprecipitation experiments have revealed that the association of NOS2 and CD74 is dependent on the caspase cleavage site, which lies in the cytosolic domain of CD74. In addition, the induction of NOS2 without NO production exerts no effect on the accumulation of CD74. This has excluded the possibility that the NOS2 protein itself prevents caspases from accessing to the cleavage site on the CD74 through steric hindrance or through inducing conformational changes in CD74. In future work, *in vitro* protein-protein interaction experiments using purified bacteria-produced fusion proteins of both NOS2 and CD74 would be useful to study whether or not CD74 interacts with NOS2.

Although the loss of NOS2 increases the cleavage of CD74 and that exogenous NO antagonises this effect in DCs, the accumulation of CD74 in LPS-induced NOS2-knockout DCs suggests that other isoforms of NOS might also be involved in the regulation of CD74 in DCs. We thus propose that NOS3 could be

such a candidate: firstly, there are only three isoforms of NO synthase, namely NOS1, NOS2 and NOS3, of which NOS1 is not expressed in DCs; secondly, the activity of NOS2 at least partially depends on NOS3; thirdly, NOS3 also binds directly to one of the antigen-presenting-related proteins, dynamin, which is subjected to cleavage by caspases (Connelly L, 2005, Vo PA 2005).

Due to the limitation of the low specificity of the NOS3 antibody and the low level of endogenous NOS3 expressed in DCs, the experiment of the co-immunoprecipitation of NOS3 with CD74 was not been carried out. Thus, the question of whether NOS3 forms a complex with CD74, as NOS2 does, has yet to be answered. Although it would be rather difficult to verify the direct interaction between NOS3 and CD74, gain-of-function and lost-of-function studies using electroploration and gene knockout have revealed that NOS3 has a similar effect on the distribution of MHC class II and on the stimulation of T cells in DCs. These results suggest that the trafficking of MHC class II to the cell surface and the stimulation of T cells in DCs are dependent on the NOS3 protein. Further studies are needed to switch from an *in vitro* study to an *in vivo* study for the identification of the potential pre-clinical significance of this caspase-NOS mediated CD74 regulation pathway. As a first step, the NOS2/NOS3 gene-modified DCs may be re-injected into the mice, together with suitable antigenic peptides followed by a monitoring of the effects on the immunoresponse of the mice, such as cytokine secretions or T cell proliferations.

In summary, in this study, we have found that CD74 is cleaved by caspases in DCs. During the maturation of DCs, the decrease of caspase activities owing to the *S*-nitrosylation by NO causes an accumulation of CD74. CD74 in

turn increases the cell surface expression of MHC class II and thus the antigen presentation ability of DCs. On the other hand, NO is mainly generated from NOS2 which is found to have a direct interaction with CD74. This direct binding protects the caspase cutting site "DQRD" in the CD74 N-terminus from caspase cleavage (Figure 40). In addition, NOS3 may also play a role in the protection of CD74. Taken together, these findings have shed new light on the post-translational regulation of CD74. Together with the current knowledge on the transcriptional and translational regulation pathway of NOS3 and NOS2, these findings may provide a convenient platform to engineer the functions of DCs and to develop or design potent vaccines against infectious diseases and cancers.

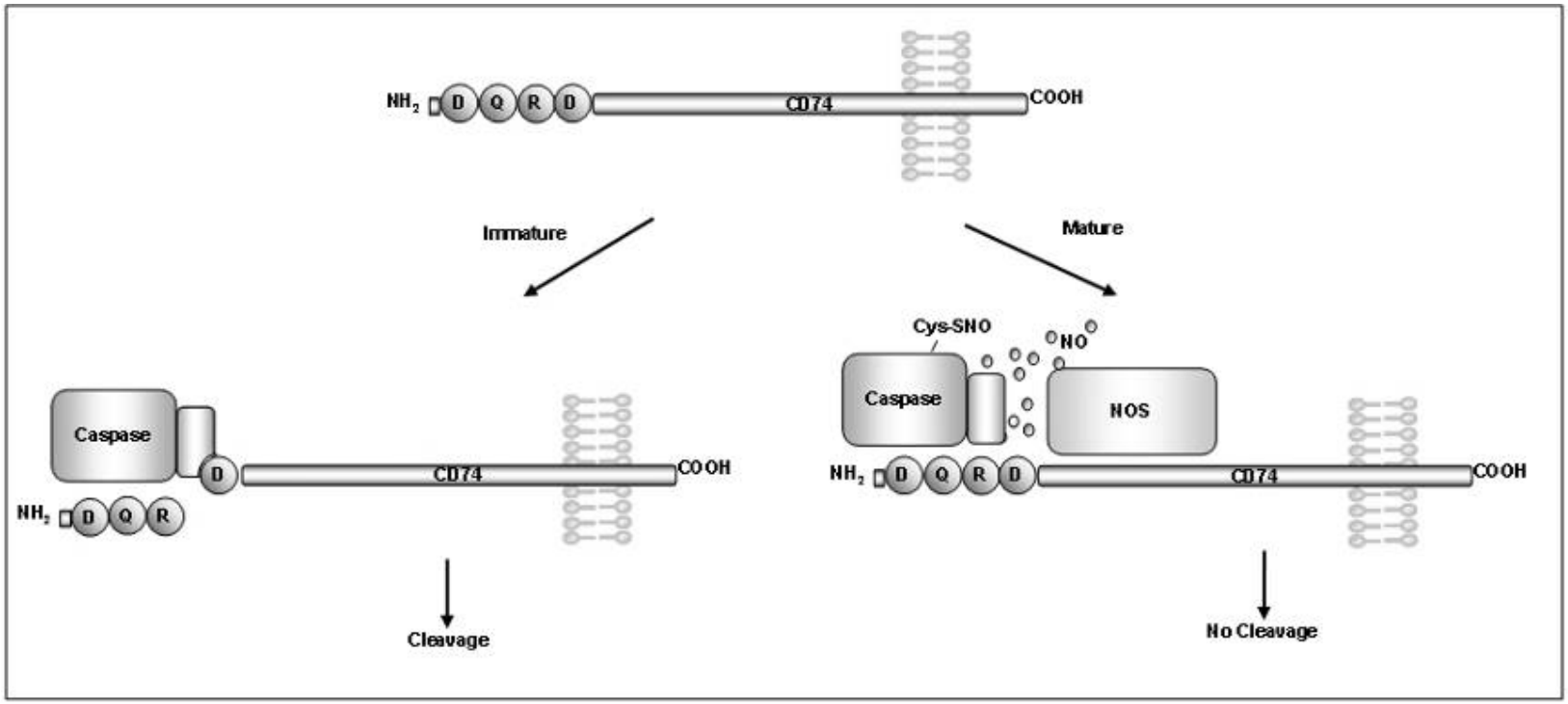


Figure 40: Model shown that NOS2 activity is essential in preventing CD74 proteolytic degradation in maturing DCs

In immature DCs, the cytoplasmic domain of CD74 is exposed to the proteolytic activity of caspases. During DC maturation, NOS2 binds to the cytoplasmic domain of CD74 and catalyses the production of NO, which in turn inhibits caspases and protects CD74 from proteolytic degradation

REFERENCE LIST

1. Accolla RS, Jotterand-Bellomo M, Scarpellino L, Maffei A, Carra G, and Guardiola J (1986) aIr-1, a Newly Found Locus on Mouse Chromosome 16 Encoding a Trans-acting Activator Factor for MHC Class II Gene Expression. *J Exp Med,* **164,** 369-374.

2. Aiello S, Noris M, Piccinini G, Tomasoni S, Casiraghi F, Bonazzola S, Mister M, Sayegh MH, and Remuzzi G (2000) Thymic Dendritic Cells Express Inducible Nitric Oxide Synthase and Generate Nitric Oxide in Response to Self- and Alloantigens. *J Immunol,* **164,** 4649-4658.

3. Ardavin C (2003) Origin, precursors and differentiation of mouse dendritic cells. *Nat Rev Immunol,* **3,** 582-590.

   Asselin-Paturel, C. (2001) Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nature immunol,* 2, 1144-1150.

4. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, and Palucka K (2000) Immunobiology of Dendritic Cells. *Annual Review of Immunology,* **18,** 767-811.

5. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, and Palucka K (2000c) Immunobiology of Dendritic Cells. *Annual Review of Immunology,* **18,** 767-811.

6. Bates TE, Loesch A, Burnstock G, and Clark JB (1996) Mitochondrial Nitric Oxide Synthase: A Ubiquitous Regulator of Oxidative Phosphorylation? *Biochemical and Biophysical Research Communications,* **218,** 40-44.

7. Becker-Herman S, Arie G, Medvedovsky H, Kerem A, and Shachar I (2005) CD74 is a member of the regulated intramembrane proteolysis-processed protein family. *Mol Biol Cell,* **16,** 5061-5069.

8. Bogdan C (2001a) Nitric oxide and the immune response. *Nat Immunol,* **2,** 907-916.

9. Bonham CA, Lu L, Li Y, Hoffman RA, Simmons RL, and Thomson AW (1996) Nitric oxide production by mouse bone marrow-derived dendritic cells: implications for the regulation of allogeneic T cell responses. *Transplantation,* **62,** 1871-1877.

10. Boss JM and Jensen PE (2003) Transcriptional regulation of the MHC class II antigen presentation pathway. *Current Opinion in Immunology,* **15,** 105-111.

11. Brigl M and Brenner MB (2004) CD1: Antigen Presentation and T Cell Function. *Annual Review of Immunology,* **22,** 817-890.

12. Cao L and Eldred WD (2001) Subcellular localization of neuronal nitric oxide synthase in turtle retina: electron immunocytochemistry. *Vis Neurosci,* **18,** 949-960.

13. Ceman S and Sant AJ (1995) The function of invariant chain in class II-restricted antigen presentation. *Seminars in Immunology,* **7,** 373-387.

14. Chang CH, Guerder S, Hong SC, van Ewijk W, and Flavell RA (1996) Mice Lacking the MHC Class II Transactivator (CIITA) Show Tissue-Specific Impairment of MHC Class II Expression. *Immunity,* **4,** 167-178.

15. Chin AI, Dempsey PW, Bruhn K, Miller JF, Xu Y, and Cheng G (2002) Involvement of receptor-interacting protein 2 in innate and adaptive immune responses. *Nature,* **416,** 190-194.

16. Chin KC, Li G, and Ting JP (1997b) Activation and transdominant suppression of MHC class II and HLA-DMB promoters by a series of C-terminal class II transactivator deletion mutants. *J Immunol,* **159,** 2789-2794.

17. Chin KC, Li G, and Ting JP (1997a) Activation and transdominant suppression of MHC class II and HLA-DMB promoters by a series of C-terminal class II transactivator deletion mutants. *J Immunol,* **159,** 2789-2794.

18. Cleeter MWJ, Cooper JM, rley-Usmar VM, Moncada S, and Schapira AHV (1994) Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide : Implications for neurodegenerative diseases. *FEBS Letters,* **345,** 50-54.

19. Clementi E, Brown GC, Feelisch M, and Moncada S (1998) Persistent inhibition of cell respiration by nitric oxide: Crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proceedings of the National Academy of Sciences,* **95,** 7631-7636.

20. Coleman JW (2001) Nitric oxide in immunity and inflammation. *International Immunopharmacology,* **1,** 1397-1406.

21. Connelly L, Madhani M, and Hobbs AJ (2005) Resistance to endotoxic shock in endothelial nitric-oxide synthase (eNOS) knock-out mice: a pro-inflammatory role for eNOS-derived no in vivo. *J Biol Chem,* **280,** 10040-10046.

22. Connelly L, Palacios-Callender M, Ameixa C, Moncada S, and Hobbs AJ (2001) Biphasic Regulation of NF-{{kappa}}B Activity Underlies the Pro- and Anti-Inflammatory Actions of Nitric Oxide. *J Immunol,* **166,** 3873-3881.

23. Connelly L, Madhani M, and Hobbs AJ (2005b) Resistance to Endotoxic Shock in Endothelial Nitric-oxide Synthase (eNOS) Knock-out Mice: A PRO-INFLAMMATORY ROLE FOR eNOS-DERIVED NO IN VIVO. *Journal of Biological Chemistry,* **280,** 10040-10046.

24. Corcoran L, Ferrero I, Vremec D, Lucas K, Waithman J, O'Keeffe M, Wu L, Wilson A, and Shortman K (2003) The lymphoid past of mouse plasmacytoid cells and thymic dendritic cells. *J Immunol,* **170,** 4926-4932.

25. Cressman DE, O'Connor WJ, Greer SF, Zhu XS, and Ting JP (2001) Mechanisms of nuclear import and export that control the subcellular localization of class II transactivator. *J Immunol,* **167,** 3626-3634.

26. Cresswell P (1994) Assembly, transport, and function of MHC class II molecules. *Annu Rev Immunol,* **12,** 259-293.

27. da Silva Correia J, Soldau K, Christen U, Tobias PS, and Ulevitch RJ (2001) Lipopolysaccharide Is in Close Proximity to Each of the Proteins in Its Membrane Receptor Complex. TRANSFER FROM CD14 TO TLR4 AND MD-2. *Journal of Biological Chemistry,* **276,** 21129-21135.

28. David A. Geller and Timothy R. Billiar (1998) Molecular biology of nitric oxide synthases *Cancer and Metastasis Reviews* **17,** 7–23.

29. de Vera ME, Shapiro RA, Nussler AK, Mudgett JS, Simmons RL, Morris SM, Jr., Billiar TR, and Geller DA (1996) Transcriptional regulation of human inducible nitric oxide

synthase (NOS2) gene by cytokines: Initial analysis of the human NOS2 promoter. *Proceedings of the National Academy of Sciences,* **93,** 1054-1059.

30. Delamarre L, Pack M, Chang H, Mellman I, and Trombetta ES (2005) Differential Lysosomal Proteolysis in Antigen-Presenting Cells Determines Antigen Fate. *Science,* **307,** 1630-1634.

31. Denzin LK and Cresswell P (1995a) HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. *Cell,* **82,** 155-165.

32. El-Sukkari D, Wilson NS, Hakansson K, Steptoe RJ, Grubb A, Shortman K, and Villadangos JA (2003) The protease inhibitor cystatin C is differentially expressed among dendritic cell populations, but does not control antigen presentation. *J Immunol,* **171,** 5003-5011.

33. Fernandez-Ruiz V, Gonzalez A, and Lopez-Moratalla N (2004a) Effect of nitric oxide in the differentiation of human monocytes to dendritic cells. *Immunol Lett,* **93,** 87-95.

34. Fiebiger E, Maehr R, Villadangos J, Weber E, Erickson A, Bikoff E, Ploegh HL, and Lennon-Dumenil AM (2002) Invariant Chain Controls the Activity of Extracellular Cathepsin L. *J Exp Med,* **196,** 1263-1270.

35. Gao S, Chen J, Brodsky SV, Huang H, Adler S, Lee JH, Dhadwal N, Cohen-Gould L, Gross SS, and Goligorsky MS (2004) Docking of Endothelial Nitric Oxide Synthase (eNOS) to the Mitochondrial Outer Membrane: a Pentabasic Amino Acid Sequence in the Autoinhibitory Domain of eNOS Targets a Progets a Proteinase K-Cleavable Peptide on the Cytoplasmic Face of Mitochondria. *Journal of Biological Chemistry,* **279,** 15968-15974.

36. Glockzin S, von Knethen A, Scheffner M, and Brune B (1999) Activation of the Cell Death Program by Nitric Oxide Involves Inhibition of the Proteasome. *Journal of Biological Chemistry,* **274,** 19581-19586.

37. Hall AV, Antoniou H, Wang Y, Cheung AH, Arbus AM, Olson SL, Lu WC, Kau C-L, Marsden PA (1996) Structural organization of the human neuronal nitric oxide synthase gene (NOS1). *J Biol Chem* **269**, 33082–33090.

38. Harris LK, McCormick J, Cartwright JE, Whitley GS, and Dash PR (2008) S-nitrosylation of proteins at the leading edge of migrating trophoblasts by inducible nitric oxide synthase promotes trophoblast invasion. *Exp Cell Res,* **314,** 1765-1776.

39. Heath WR and Carbone FR (2001) Cross-Presentation, Dendritic CElls, Tolerance and Immunity. *Annual Review of Immunology,* **19,** 47-64.

40. Hickey MJ, Sharkey KA, Sihota EG, Reinhardt PH, Macmicking JD, Nathan C, and Kubes P (1997) Inducible nitric oxide synthase-deficient mice have enhanced leukocyte-endothelium interactions in endotoxemia. *FASEB J,* **11,** 955-964.

41. Hideki Nakano, Manabu Yanagita, and Michael Dee Gunn (2001) Cd11c+B220+Gr-1+ Cells in Mouse Lymph Nodes and Spleen Display Characteristics of Plasmacytoid Dendritic Cells. *J Exp Med,* **194**, 1171–1178

42. Hiscott J, Lin R, Nakhaei P, and Paz S (2006) MasterCARD: a priceless link to innate immunity. *Trends Mol Med,* **12,** 53-56.

43. Hofmann K, Bucher P, and Tschopp J (1997) The CARD domain: a new apoptotic signalling motif. *Trends in Biochemical Sciences,* **22,** 155-156.

44. Honey K and Rudensky AY (2003) Lysosomal cysteine proteases regulate antigen presentation. *Nat Rev Immunol,* **3,** 472-482.

45. Hsing LC and Rudensky AY (2005) The lysosomal cysteine proteases in MHC class II antigen presentation. *Immunol Rev,* **207,** 229-241.

46. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, and Steinman RM (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med,* **176,** 1693-1702.

47. Inohara N, Koseki T, del PL, Hu Y, Yee C, Chen S, Carrio R, Merino J, Liu D, Ni J, and Nunez G (1999) Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. *J Biol Chem,* **274,** 14560-14567.

48. Inohara N, Ogura Y, Chen FF, Muto A, and Nunez G (2001) Human Nod1 confers responsiveness to bacterial lipopolysaccharides. *J Biol Chem,* **276,** 2551-2554.

49. Isenberg JS (2003) Inhibition of nitric oxide synthase (NOS) conversion of L-arginine to nitric oxide (NO) decreases low density mononuclear cell (LD MNC) trans-endothelial migration and cytokine output. *J Surg Res,* **114,** 100-106.

50. Jaksits S, Bauer W, Kriehuber E, Zeyda M, Stulnig TM, Stingl G, Fiebiger E, and Maurer D (2004) Lipid Raft-Associated GTPase Signaling Controls Morphology and CD8+ T Cell Stimulatory Capacity of Human Dendritic Cells. *J Immunol,* **173,** 1628-1639.

51. Kang BH, Plescia J, Dohi T, Rosa J, Doxsey SJ, and Altieri DC (2007) Regulation of Tumor Cell Mitochondrial Homeostasis by an Organelle-Specific Hsp90 Chaperone Network. *Cell,* **131,** 257-270.

52. Katagiri T, Takahashi T, Sasaki T, Nakamura S, and Hattori S (2000) Protein-tyrosine kinase Pyk2 is involved in interleukin-2 production by Jurkat T cells via its tyrosine 402. *J Biol Chem,* **275,** 19645-19652.

53. Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, Tsujimura T, Takeda K, Fujita T, Takeuchi O, and Akira S (2005) Cell type-specific involvement of RIG-I in antiviral response. *Immunity,* **23,** 19-28.

54. Ke S, Chen XH, Li H, Li JF, Gu QL, Liu BY, and Zhu ZG (2007) Silencing invariant chain of DCs enhances Th1 response using small interfering RNA. *Cell Biology International,* **31,** 663-671.

55. Kishimoto J, Spurr N. Liao M, Lizhi L, Emson P, Xu W (1992) Localization of brain nitric oxide synthase (NOS) to human chromosome 12. *Genomics* **14**, 802–804

56. King SB (2005) N-hydroxyurea and acyl nitroso compounds as nitroxyl (HNO) and nitric oxide (NO) donors. *Curr Top Med Chem,* **5,** 665-673.

57. Kobzik L, Stringer B, Balligand JL, Reid MB, and Stamler JS (1995) Endothelial-Type Nitric Oxide Synthase (ec-NOS) in Skeletal Muscle Fibers: Mitochondrial Relationships. *Biochemical and Biophysical Research Communications,* **211,** 375-381.

58. Kone BC, Kuncewicz T, Zhang W, and Yu ZY (2003) Protein interactions with nitric oxide synthases: controlling the right time, the right place, and the right amount of nitric oxide. *Am J Physiol Renal Physiol,* **285,** F178-F190.

59. Kropshofer H, Spindeldreher S, Rohn TA, Platania N, Grygar C, Daniel N, Wolpl A, Langen H, Horejsi V, and Vogt AB (2002) Tetraspan microdomains distinct from lipid rafts enrich select peptide-MHC class II complexes. *Nat Immunol,* **3,** 61-68.

60. Kurts C (2006) Dendritic cells: not just another cell type in the kidney, but a complex immune sentinel network. *Kidney Int,* **70,** 412-414.

61. Lau AH and Thomson AW (2003) Dendritic cells and immune regulation in the liver. *Gut,* **52,** 307-314.

62. Leibundgut-Landmann S, Waldburger JM, Krawczyk M, Otten LA, Suter T, Fontana A, cha-Orbea H, and Reith W (2004) Mini-review: Specificity and expression of CIITA, the master regulator of MHC class II genes. *Eur J Immunol,* **34,** 1513-1525.

63. LeibundGut-Landmann S, Waldburger JM, Reis e Sousa, cha-Orbea H, and Reith W (2004) MHC class II expression is differentially regulated in plasmacytoid and conventional dendritic cells. *Nat Immunol,* **5,** 899-908.

64. Leng L, Metz CN, Fang Y, Xu J, Donnelly S, Baugh J, Delohery T, Chen Y, Mitchell RA, and Bucala R (2003) MIF Signal Transduction Initiated by Binding to CD74. *J Exp Med,* **197,** 1467-1476.

65. Leon B, Lopez-Bravo M, and Ardavin C (2007) Monocyte-Derived Dendritic Cells Formed at the Infection Site Control the Induction of Protective T Helper 1 Responses against Leishmania. *Immunity,* **26,** 519-531.

66. Li WP, Liu P, Pilcher BK, and Anderson RG (2001) Cell-specific targeting of caveolin-1 to caveolae, secretory vesicles, cytoplasm or mitochondria. *J Cell Sci,* **114,** 1397-1408.

67. Liu K, Waskow C, Liu X, Yao K, Hoh J, and Nussenzweig M (2007) Origin of dendritic cells in peripheral lymphoid organs of mice. *Nat Immunol,* **8,** 578-583.

68. Liu S, An H, Li N, Yu Y, Lin N, Wan T, Zhang M, Wang W, and Cao X (2003) Cloning and identification of a novel human ubiquitin-like protein, DC-UbP, from dendritic cells. *Biochemical and Biophysical Research Communications,* **300,** 800-805.

69. Lo HP, ckland-Berglund CE, Pritchard KA, Jr., Guice KS, and Oldham KT (2001) Attenuated expression of inducible nitric oxide synthase in lung microvascular endothelial cells is associated with an increase in ICAM-1 expression. *J Pediatr Surg,* **36,** 1136-1142.

70. Lu L, Bonham CA, Chambers FG, Watkins SC, Hoffman RA, Simmons RL, and Thomson AW (1996) Induction of nitric oxide synthase in mouse dendritic cells by IFN-gamma, endotoxin, and interaction with allogeneic T cells: nitric oxide production is associated with dendritic cell apoptosis. *J Immunol,* **157,** 3577-3586.

71. Lu L, Woo J, Rao AS, Li Y, Watkins SC, Qian S, Starzl TE, Demetris AJ, and Thomson AW (1994) Propagation of dendritic cell progenitors from normal mouse liver using granulocyte/macrophage colony-stimulating factor and their maturational development in the presence of type-1 collagen. *J Exp Med,* **179,** 1823-1834.

72. Luschen S, Ussat S, Kronke M, and dam-Klages S (1998b) Cleavage of human cytosolic phospholipase A2 by caspase-1 (ICE) and caspase-8 (FLICE). *Biochem Biophys Res Commun,* **253,** 92-98.

73. Luschen S, Ussat S, Kronke M, and dam-Klages S (1998a) Cleavage of human cytosolic phospholipase A2 by caspase-1 (ICE) and caspase-8 (FLICE). *Biochem Biophys Res Commun,* **253,** 92-98.

74. Maehr R, Hang HC, Mintern JD, Kim YM, Cuvillier A, Nishimura M, Yamada K, Shirahama-Noda K, Hara-Nishimura I, and Ploegh HL (2005) Asparagine endopeptidase is not essential for class II MHC antigen presentation but is required for processing of cathepsin L in mice. *J Immunol,* **174,** 7066-7074.

75. Malissen B, Price MP, Goverman JM, McMillan M, White J, Kappler J, Marrack P, Pierres A, Pierres M, and Hood L (1984) Gene transfer of H-2 class II genes: antigen presentation by mouse fibroblast and hamster B-cell lines. *Cell,* **36,** 319-327.

76. Maniscalco M, Sofia M, and Pelaia G (2007) Nitric oxide in upper airways inflammatory diseases. *Inflamm Res,* **56,** 58-69.

77. Manoury B, Mazzeo D, Li DN, Billson J, Loak K, Benaroch P, and Watts C (2003a) Asparagine endopeptidase can initiate the removal of the MHC class II invariant chain chaperone. *Immunity,* **18,** 489-498.

78. Marshall He, Merchant Kuna, and Stamler JS (2000) Nitrosation and oxidation in the regulation of gene expression. *FASEB J,* **14,** 1889-1900.

79. Masternak K and Reith W (2002a) Promoter-specific functions of CIITA and the MHC class II enhanceosome in transcriptional activation. *EMBO J,* **21,** 1379-1388.

80. Matza D, Kerem A, and Shachar I (2003) Invariant chain, a chain of command. *Trends Immunol,* **24,** 264-268.

81. Matjaž Jeras (2005) *In vitro* preparation and functional assessment of human monocyte-derived dendritic cells—potential antigen specific modulators of *in vivo* immune responses. *Transplant Immunology.* **14**, 231-244.

82. McWhirter SM, Tenoever BR, and Maniatis T (2005) Connecting mitochondria and innate immunity. *Cell,* **122,** 645-647.

80. Mellgren RL Detergent-resistant membrane subfractions containing proteins of plasma membrane, mitochondrial, and internal membrane origins. *Journal of Biochemical and Biophysical Methods,* **In Press, Corrected Proof**.

81. Mellman I, Turley SJ, and Steinman RM (1998) Antigen processing for amateurs and professionals. *Trends Cell Biol,* **8,** 231-237.

82. Mempel TR, Henrickson SE, and von Andrian UH (2004) T-cell priming by dendriticcells in lymph nodes occurs in three distinct phases. *Nature,* **427,** 154-159.

83. Miguel A. Tam and Mary Jo Wick (2004) Dendritic cells and immunity to Listeria: TipDCs are a new recruit *Trends in Immunology*, **25**, 335-339.

84. Miller MR and Megson IL (2007) Recent developments in nitric oxide donor drugs. Br J *Pharmacol,* **151,** 305-321.

85. Muhlethaler-Mottet A, Otten LA, Steimle V, and Mach B (1997a) Expression of MHC class II molecules in different cellular and functional compartments is controlled by differential usage of multiple promoters of the transactivator CIITA. *EMBO J,* **16,** 2851-2860.

86. Muhlethaler-Mottet A, Otten LA, Steimle V, and Mach B (1997b) Expression of MHC class II molecules in different cellular and functional compartments is controlled by differential usage of multiple promoters of the transactivator CIITA. *EMBO J,* **16,** 2851-2860.

87. Mukarkami H, Akbar SMF, Matsui H, Horiike N, and Onji M (2002) Macrophage migration inhibitory factor activates antigen-presenting dendritic cells and induces inflammatory cytokines in ulcerative colitis. *Clinical & Experimental Immunology,* **128,** 504-510.

88. Nakagawa TY, Brissette WH, Lira PD, Griffiths RJ, Petrushova N, Stock J, McNeish JD, Eastman SE, Howard ED, Clarke SR, Rosloniec EF, Elliott EA, and Rudensky AY (1999) Impaired invariant chain degradation and antigen presentation and diminished collagen-induced arthritis in cathepsin S null mice. *Immunity,* **10,** 207-217.

89. Natasa K. The role of cystatins in cells of the immune system. FEBS letters 580[27], 6295-6301. 2006.

90. Natalya V. Serbina (2003) TNF/iNOS-Producing Dendritic Cells Mediate Innate Immune Defense against Bacterial Infection. *Immunity*, **9**, 59–70

91. Navarro-Lerida I, varez-Barrientos A, and Rodriguez-Crespo I (2006) N-terminal palmitoylation within the appropriate amino acid environment conveys on NOS2 the ability to progress along the intracellular sorting pathways. *J Cell Sci,* **119,** 1558-1569.

92. Nickerson K, Sisk TJ, Inohara N, Yee CS, Kennell J, Cho MC, Yannie PJ, Nunez G, and Chang CH (2001g) Dendritic cell-specific MHC class II transactivator contains a caspase recruitment domain that confers potent transactivation activity. *J Biol Chem,* **276,** 19089-19093.

93. Niedbala W, Wei XQ, Campbell C, Thomson D, Komai-Koma M, and Liew FY (2002) Nitric oxide preferentially induces type 1 T cell differentiation by selectively up-regulating IL-12 receptor beta 2 expression via cGMP. *Proc Natl Acad Sci U S A,* **99,** 16186-16191.

94. Nikolic T, de Bruijn MF, Lutz MB, and Leenen PJ (2003) Developmental stages of myeloid dendritic cells in mouse bone marrow. *Int Immunol,* **15,** 515-524.

95. O'Sullivan DM, Noonan D, and Quaranta V (1987) Four Ia invariant chain forms derive from a single gene by alternate splicing and alternate initiation of transcription/translation. *J Exp Med,* **166,** 444-460.

96. Panjwani NN, Popova L, and Srivastava PK (2002) Heat shock proteins gp96 and hsp70 activate the release of nitric oxide by APCs. *J Immunol,* **168,** 2997-3003.

97. Paolucci C, Burastero SE, Rovere-Querini P, De Palma C, Falcone S, Perrotta C, Capobianco A, Manfredi AA, and Clementi E (2003) Synergism of nitric oxide and maturation signals on human dendritic cells occurs through a cyclic GMP-dependent pathway. *J Leukoc Biol,* **73,** 253-262.

98. Pierre P and Mellman I (1998) Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. *Cell,* **93,** 1135-1145.

99. Pierre P, Shachar I, Matza D, Gatti E, Flavell RA, and Mellman I (2000) Invariant chain controls H2-M proteolysis in mouse splenocytes and dendritic cells. *J Exp Med,* **191,** 1057-1062.

100. Poloso NJ, Denzin LK, and Roche PA (2006) CDw78 Defines MHC Class II-Peptide Complexes That Require Ii Chain-Dependent Lysosomal Trafficking, Not Localization to a Specific Tetraspanin Membrane Microdomain. *J Immunol,* **177,** 5451-5458.

101. Pond L, Kuhn LA, Teyton L, Schutze MP, Tainer JA, Jackson MR, and Peterson PA (1995) A Role for Acidic Residues in Di-leucine Motif-based Targeting to the Endocytic Pathway. *Journal of Biological Chemistry,* **270,** 19989-19997.

102. Randolph GJ (2001) Dendritic cell migration to lymph nodes: cytokines, chemokines, and lipid mediators. *Semin Immunol,* **13,** 267-274.

103. Randolph GJ, Inaba K, Robbiani DF, Steinman RM, and Muller WA (1999) Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. *Immunity,* **11,** 753-761.

104. Randolph GJ, Sanchez-Schmitz G, Liebman RM, and Schakel K (2002) The CD16(+) (FcγRIII(+)) subset of human monocytes preferentially becomes migratory dendritic cells in a model tissue setting. *J Exp Med,* **196,** 517-527.

105. Ratovitski EA, Bao C, Quick RA, McMillan A, Kozlovsky C, and Lowenstein CJ (1999) An Inducible Nitric-oxide Synthase (NOS)-associated Protein Inhibits NOS Dimerization and Activity. *Journal of Biological Chemistry,* **274,** 30250-30257.

106. Raval A, Weissman JD, Howcroft TK, and Singer DS (2003) The GTP-binding domain of class II transactivator regulates its nuclear export. *J Immunol,* **170,** 922-930.

107. Rechsteiner M and Rogers SW (1996) PEST sequences and regulation by proteolysis. *Trends Biochem Sci,* **21,** 267-271.

108. Reith W and Mach B (2001b) The bare lymphocyte syndrome and the regulation of MHC expression. *Annual Review of Immunology,* **19,** 331-373.

109. Riese RJ, Wolf PR, Bromme D, Natkin LR, Villadangos JA, Ploegh HL, and Chapman HA (1996) Essential role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading. *Immunity,* **4,** 357-366.

110. Riese RJ and Chapman HA (2000) Cathepsins and compartmentalization in antigen presentation. *Current Opinion in Immunology,* **12,** 107-113.

111. Rissoan MC, Duhen T, Bridon JM, driss-Vermare N, Peronne C, de S, V, Briere F, and Bates EE (2002) Subtractive hybridization reveals the expression of immunoglobulin-like transcript 7, Eph-B1, granzyme B, and 3 novel transcripts in human plasmacytoid dendritic cells. *Blood,* **100,** 3295-3303.

112. Rogers S, Wells R, and Rechsteiner M (1986) Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science,* **234,** 364-368.

113. Rohn TA, Boes M, Wolters D, Spindeldreher S, Muller B, Langen H, Ploegh H, Vogt AB, and Kropshofer H (2004) Upregulation of the CLIP self peptide on mature dendritic cells antagonizes T helper type 1 polarization. *Nat Immunol,* **5,** 909-918.

114. Sallusto, F. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by GMSF plus IL-4 and downregulated by TNF alpha. *J.exp. Me,* **179**, 1109-1118

115. Santambrogio L, Potolicchio I, Fessler SP, Wong SH, Raposo G, and Strominger JL (2005) Involvement of caspase-cleaved and intact adaptor protein 1 complex in endosomal remodeling in maturing dendritic cells. *Nat Immunol,* **6,** 1020-1028.

116. Santambrogio L, Sato AK, Fischer FR, Dorf ME, and Stern LJ (1999) Abundant Empty Class II MHC Molecules on the Surface of Immature Dendritic Cells. *Proceedings of the National Academy of Sciences,* **96,** 15050-15055.

117. Schnappauf F, Hake SB, Camacho Carvajal MM, Bontron S, Lisowska-Grospierre B, and Steimle V (2003) N-terminal Destruction Signals Lead to Rapid Degradation of the Major Histocompatibility Complex Class II Transactivator CIITA. *Eur J Immunol,* **33,** 2337-2347.

118. Schuman EM, Madison DV (1991) A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science* **254**, 1503–1506.

119. Serbina NV, Jia T, Hohl TM, and Pamer EG (2008) Monocyte-Mediated Defense Against Microbial Pathogens. *Annual Review of Immunology,* **26**.

120. Seth RB, Sun L, Ea CK, and Chen ZJ (2005) Identification and Characterization of MAVS, a Mitochondrial Antiviral Signaling Protein that Activates NF-κB and IRF 3. *Cell,* **122,** 669-682.

121. Shi GP, Villadangos JA, Dranoff G, Small C, Gu L, Haley KJ, Riese R, Ploegh HL, and Chapman HA (1999) Cathepsin S Required for Normal MHC Class II Peptide Loading and Germinal Center Development. *Immunity,* **10,** 197-206.

122. Shi X, Leng L, Wang T, Wang W, Du X, Li J, McDonald C, Chen Z, Murphy JW, Lolis E, Noble P, Knudson W, and Bucala R (2006) CD44 Is the Signaling Component of the Macrophage Migration Inhibitory Factor-CD74 Receptor Complex. *Immunity,* **25,** 595-606.

123. Shin JS, Ebersold M, Pypaert M, Delamarre L, Hartley A, and Mellman I (2006) Surface expression of MHC class II in Dendritic Cells is Controlled by Regulated Ubiquitination. *Nature,* **444,** 115-118.

124. Shortman K and Naik SH (2007) Steady-state and Inflammatory Dendritic-cell Development. *Nat Rev Immunol,* **7,** 19-30.

125. Shurin GV, Tourkova IL, Chatta GS, Schmidt G, Wei S, Djeu JY, and Shurin MR (2005) Small Rho GTPases Regulate Antigen Presentation in Dendritic Cells. *J Immunol,* **174,** 3394-3400.

126. Silacci P, Mottet A, Steimle V, Reith W, and Mach B (1994) Developmental Extinction of Major Histocompatibility Complex Class II Gene Expression in Plasmocytes is Mediated by Silencing of the Transactivator Gene CIITA. *J Exp Med,* **180,** 1329-1336.

127. Spits H, Couwenberg F, Bakker AQ, Weijer K, and Uittenbogaart CH (2000) Id2 and Id3 Inhibit Development of CD34+ Stem Cells into Predendritic Cell (Pre-DC)2 but Not into Pre-DC1: Evidence for a Lymphoid Origin of Pre-DC2. *J Exp Med,* **192,** 1775-1784.

128 Steimle V, Siegrist CA, Mottet A, Lisowska-Grospierre B, and Mach B (1994) Regulation of MHC Class II Expression by Interferon-gamma Mediated by the Transactivator Gene CIITA. *Science,* **265,** 106-109.

129. Steimle V, Otten LA, Zufferey M, and Mach B (2007) Pillars Article: Complementation Cloning of an MHC Class II Transactivator Mutated in Hereditary MHC Class II Deficiency (or Bare Lymphocyte Syndrome). Cell 1993. 75: 135-146. *J Immunol,* **178,** 6677-6688.

130. Tai AKF, Zhou G, Chau Ky, and Ono SJ (1999) Cis-element Dependence and Occupancy of the Human Invariant Chain Promoter in CIITA-Dependent and -independent Transcription. *Molecular Immunology,* **36,** 447-460.

131. Takeda K, Kaisho T, and Akira S (2003) Toll-like Receptors. *Annual Review of Immunology,* **21,** 335-376.

132. Tatoyan A and Giulivi C (1998) Purification and Characterization of a Nitric-oxide Synthase from Rat Liver Mitochondria. *Journal of Biological Chemistry,* **273,** 11044-11048.

133. Ting JP and Davis BK (2005) CATERPILLER: a Novel Gene Family Important in Immunity, Cell Death, and Diseases. *Annu Rev Immunol,* **23,** 387-414.

134. Ting JP-Y and Trowsdale J (2002) Genetic Control of MHC Class II Expression. *Cell,* **109,** S21-S33.

135. Towey M and Kelly AP (2002) Nuclear localisation of CIITA is Controlled by a Carboxy Terminal Leucine-rich Repeat Region. *Molecular Immunology,* **38,** 627-634.

136. Trombetta ES and Mellman I (2005) CELL Biology of Antigen Processing *in vitro* and *in vivo*. *Annual Review of Immunology,* **23,** 975-1028.

137. Tsan MF and Gao B (2004a) Endogenous Ligands of Toll-like Receptors. *J Leukoc Biol,* **76,** 514-519.

138 Tsan MF and Gao B (2004b) Heat Shock Protein and Innate Immunity. *Cell Mol Immunol,* **1,** 274-279.

139. Turley SJ, Inaba K, Garrett WS, Ebersold M, Unternaehrer J, Steinman RM, and Mellman I (2000) Transport of Peptide-MHC Class II Complexes in Developing Dendritic Cells. *Science,* **288,** 522-527.

140. Turnbull E and MacPherson G (2001) Immunobiology of Dendritic Cells in the Rat. *Immunological Reviews,* **184,** 58-68.

141. Van den Elsen PJ, van der Stoep N, Vietor HE, Wilson L, van Zutphen M, and Gobin SJP (2000) Lack of CIITA Expression is Central to the Absence of Antigen Presentation Functions of Trophoblast Cells and is Caused by methylation of the IFN-γ Inducible Promoter (PIV) of CIITA. *Human Immunology,* **61,** 850-862.

142. Van Niel G, Wubbolts R, ten Broeke T, Buschow SI, Ossendorp FA, Melief CJ, Raposo G, van Balkom BW, and Stoorvogel W (2006) Dendritic Cells Regulate Exposure of MHC Class II at Their Plasma Membrane by Oligoubiquitination. *Immunity,* **25,** 885-894.

143. Villadangos JA, Cardoso M, Steptoe RJ, van BD, Pooley J, Carbone FR, and Shortman K (2001a) MHC Class II Expression is Regulated in Dendritic Cells Independently of Invariant Chain Degradation. *Immunity,* **14,** 739-749.

144. Villadangos JA, Heath WR, and Carbone FR (2007) Outside Looking in: the Inner Workings of the Cross-presentation Pathway within Dendritic Cells. *Trends Immunol,* **28,** 45-47.

145. Villadangos JA and Ploegh HL (2000) Proteolysis in MHC Class II Antigen Presentation: Who's in Charge? *Immunity,* **12,** 233-239.

146. Villadangos JA and Schnorrer P (2007a) Intrinsic and Cooperative Antigen-presenting Functions of Dendritic-cell Subsets *in vivo*. *Nat Rev Immunol,* **7,** 543-555.

147. Viville S, Neefjes J, Lotteau V, Dierich A, Lemeur M, Ploegh H, Benoist C, and Mathis D (1993) Mice Lacking the MHC class II-associated Invariant Chain. *Cell,* **72,** 635-648.

148. Vo PA, Lad B, Tomlinson JA, Francis S, and Ahluwalia A (2005) Autoregulatory Role of Endothelium-derived Nitric Oxide (NO) on Lipopolysaccharide-induced Vascular Inducible NO Synthase Expression and Function. *J Biol Chem,* **280,** 7236-7243.

149. Weis M, Schlichting CL, Engleman EG, and Cooke JP (2002) Endothelial Determinants of Dendritic Cell Adhesion and Migration: New Implications for Vascular Diseases. *Arterioscler Thromb Vasc Biol,* **22,** 1817-1823.

150. Wilson NS and Villadangos JA (2005). Regulation of Antigen Presentation and Cross-Presentation in the Dendritic Cell Network: Facts, Hypothesis, and Immunological Implications. In Frederick,W.A. (Ed.), *Advances in Immunology*, . Academic Press, pp. 241-305.

151. Wong AW, Brickey WJ, Taxman DJ, van Deventer HW, Reed W, Gao JX, Zheng P, Liu Y, Li P, Blum JS, McKinnon KP, and Ting JPY (2003) CIITA-Regulated Plexin-A1 Affects T-cell-Dendritic Cell Interactions. *Nat Immunol,* **4,** 891-898.

152. Wong SH, Santambrogio L, and Strominger JL (2004) Caspases and Nitric Oxide Broadly Regulate Dendritic Cell Maturation and Surface Expression of Class II MHC Proteins. *Proc Natl Acad Sci U S A,* **101,** 17783-17788.

153. Wu YP, Siao CJ, Lu W, Sung TC, Frohman MA, Milev P, Bugge TH, Degen JL, Levine JM, Margolis RU, and Tsirka SE (2000) The Tissue Plasminogen Activator (tPA)/Plasmin Extracellular Proteolytic System regulates Seizure-Induced Hippocampal Mossy Fiber Outgrowth Through a proteoglycan substrate. *J Cell Biol,* **148,** 1295-1304.

154. Xie QW, Kashiwabara Y, and Nathan C (1994) Role of Transcription Factor NF-kappa B/Rel in Induction of Nitric Oxide Synthase. *Journal of Biological Chemistry,* **269,** 4705-4708.

155. Xu Y, Harton JA, and Smith BD (2008) CIITA Mediates Interferon-*γ* Repression of Collagen Transcription through Phosphorylation-dependent Interactions with Co-repressor Molecules. *Journal of Biological Chemistry,* **283,** 1243-1256.

156. Yang Y, Liang Y, Qu L, Chen Z, Yi M, Li K, and Lemon SM (2007) Disruption of Innate Immunity due to Mitochondrial Targeting of a Picornaviral Protease Precursor. *Proc Natl Acad Sci U S A,* **104,** 7253-7258.

157. Yee CSK, Yao Y, Xu Q, McCarthy B, Sun-Lin D, Tone M, Waldmann H, and Chang CH (2005) Enhanced Production of IL-10 by Dendritic Cells Deficient in CIITA. *J Immunol,* **174,** 1222-1229.

158. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, and Fujita T (2004) The RNA Helicase RIG-I has an Essential Function in Double-Stranded RNA-Induced Innate Antiviral Responses. *Nat Immunol,* **5,** 730-737.

159. Zhu L and Jones PP (1990) Transcriptional Control of the Invariant Chain Gene Involves Promoter and Enhancer Elements Common to and Distinct from Major Histocompatibility Complex Class II Genes. *Mol Cell Biol,* **10,** 3906-3916.

160. Zhu XS, Linhoff MW, Li G, Chin KC, Maity SN, and Ting JP-Y (2000) Transcriptional Scaffold: CIITA Interacts with NF-Y, RFX, and CREB To Cause Stereospecific Regulation of the Class II Major Histocompatibility Complex Promoter. *Mol Cell Biol,* **20,** 6051-6061.

PUBLICATIONS AND CONFERENCES

1. **Huang D.**, Sylvia Lim, Rong YR Chua, Ng ML and Wong SH. A novel splice-isoform of the MHC class II transactivator regulates nitric oxide synthesis and antigen presentation in maturing dendritic cells. *Journal of Immunology*, (Submitted).
2. Tan, YR; Chiow KH, **Huang D.,** and Wong SH, Andrographolide regulates epidermal growth factor receptor (EGFR) trafficking in epidermoid carcinoma A-431 cells. *British Journal of Pharmacology* (*In press*)
3. Ho YH, Cai DT, **Huang D.**, Wang CC, Wong SH. Caspases regulate VAMP-8 expression and phagocytosis in dendritic cells.*Biochem Biophys Res Commun*. 2009 387(2):371-375.
4. Ho YH, Cai DT, Wang CC, **Huang D.**, and Wong SH., Vesicle-associated membrane protein-8/enodbrevin negatively regulates phagocytosis of bacteria in dendritic cells. *Journal of Immunology*, 2008 180 (5): 1431-1440.
5. **Huang D.,** Cai DT, Chua RY, Kemeny DM, and Wong SH., Nitric oxide synthetase-2 interacts with CD74 and inhibits Its cleavage by caspase during dendritic cell development. *Journal of Biological Chemistry*, 2008 283 (3):1713-1722.
6. **Huang D.**, and Wong SH., Nitric oxide alone promotes maturation of dendritic cells. *1st International-Singapore Immunology Symposium*, 2008.